Prevalence of Autoplaque Phenomenon in Bacterial Communities from Spatially Heterogeneous Environment of an Abandoned Mine

Swagata Karmakar, Vandana Mishra and Radhey Shyam Sharma*

Department of Environmental Studies, Bio resources and Environmental Biotechnology Laboratory, University of Delhi, Delhi-110 007, India

*Corresponding author

ABSTRACT

The autoplaque or autolysis of bacterial cell influences the evolutionary potential of clinically important bacteria. It serves as a selection pressure on a bacterial community and provides social goods (nutrients, secondary metabolites, free DNA) in environment. Therefore, it has a major impact on the ecological fitness of microbial communities; however its prevalence in microbial communities of degraded ecosystems is not known. Therefore, we purified bacterial communities from spatially distributed soil (10m scale) and those associated with soil particle size fractions (gravel, sand, silt and clay) of abandoned mine and assessed the prevalence of autoplaque phenomenon. To our knowledge, this is the first report on the occurrence of autoplaques phenomenon in the members of Micrococccaeae. The study reports the microbial communities of degraded mine possess a substantial level of autoplaque phenomenon. It was found that in spite of taxonomic closeness, the particle-associated bacterial communities differ in their autoplaque properties. However, the spatially distributed bacterial communities showed a similar autoplaque property. The in-depth analyses of these bacterial communities will help to identify environmental and physiological factors affecting the autoplaque phenomenon. Such studies would be useful to improve the microbial technologies for environmental restoration of degraded ecosystems.

Keywords
Autoplaque, Bacterial autolysis, Microbial ecology, Spatial ecology, Abandoned mine, Soil particle.

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Introduction

The autoplaque phenomenon has been intriguing microbiologists for more than five decades (Berk, 1963; Krylov et al., 2016). The autoplaque phenomenon is characterized by the spontaneous appearance of plaques on the bacterial lawn without exposure to any external phage. Though, autolysis has been thoroughly investigated in Pseudomonas aeruginosa (Berk and Kazanas, 1963; Shaburova et al., 2001) it has also been described in several bacteria genera, such as Bacillus cereus (McCloy, 1958), B. subtilis (Loessner et al., 1997), Brucella abortus (Renoux and Suire, 1963), Fibrobacter succinogenes (Singh et al., 2003), Myxococcus xanthus (Wireman and Dworkin, 1977; Breyen and Dworkin, 1984), Neisseria gonorrhoeae (Campbell et al., 1985), Nodularia spumigena (Jenkins and Hayes, 2006) and Rhizobium trifolii (Barnet and Vincent, 1969).

Other microbes, like yeast, also show autolysis (Lindegren and Bang, 1961).
However, the biology and ecology of the autoplaque phenomenon differ in these bacteria.

The autoplaque phenomenon is suggested as a life-style of bacterial species, where bacterial genes are programmed to trigger autolysis (programmed cell death) in a bacterial cell(s). However, other factors inducing autoplaque are also known. In *P. aeruginosa*, it is linked to a life-style of pathogenic variants. In *M. xanthus*, autolysis is developmentally regulated and found essential during formation of multicellular fruiting bodies (Wireman and Dworkin, 1977; Rosenbluh and Rosenberg, 1990). The most important factor responsible for autoplaque formation is through induction of prophage present in the bacterial genome and a complex interaction of bacterial genotype with the environment. The prophage induced autoplaque phenomenon has been shown in *B. cereus* (McCloy, 1958), *B. subtilis* (Loessner et al., 1997), *Escherichia coli* (Jacob and Wollman, 1960), *P. aeruginosa* (Shaburova et al., 2001), *Nodularia succinogenes* (Jenkins and Hayes, 2006). In fact, an evolutionary link between a prophage and differentiation in *B. subtilis* has been suggested, however, the underlying mechanisms and associated factors are still not fully understood (Lewis et al., 1998; Luo et al., 2012). Besides this, the mutations shifting the balance from lysogeny to lysis have also been suggested as a cause of autoplaque phenomenon (Bednarz et al., 2014).

Exposure to lethal factors, such as chemical toxins, mutagens, oxidants, heat has also been reported to trigger autolysis (Lewis, 2000). The cell density, nutrient levels, bactericidal and genotoxic chemicals (antibiotics, sulfa drugs, and acridines), chelating agents and divalent cations, temperature and agar base also have a profound influence on the autoplaque phenomenon (Holloway, 1969; Campbell et al., 1985). In *B. subtilis*, the lack of carbon source triggers autoplaque, whereas, in *Fibrobacter succinogenes*, withdrawal of sugar inhibits autoplaque formation. In *M. xanthus*, though it is developmentally regulated, the glucosamine and fatty acid trigger the autolysis. Some phages show autoplaques of remarkably similar characteristics (Holloway, 1969); however, some bacteria show autoplaques of different size and shape (Campbell et al., 1985; Shaburova et al., 2001). In fact, the frequency of autoplaques also varies with the levels of light and nutrient during culture conditions (Breyen and Dworkin, 1984).

Emerging evidence suggests that auto cell lysis is a result of a biological response to intracellular or population level signal (quorum sensing). For example, autolysis in mother cell provides nutrition and energy to the sporulating daughter cells (*Myxococcus spp.* and *Bacillus subtilis*) (Wireman and Dworkin, 1975; Wireman and Dworkin, 1977; Doi 1989, Rosenbluh and Rosenberg, 1990). Autolysis increases the adaptive advantage of the bacteria to survive under diverse stresses through an influence on various phenotypic traits (mucoid production, biofilm development, chloramphenicol tolerance) (Holloway, 1969; Zierdt, 1971; Webb et al., 2003; Rakhimova 2007).

In fact, in some bacteria, the autoplaque forming phages show a narrow host range and serve as a vehicle to specifically drive genetic diversification of the bacterial host (Luo et al., 2012). Further, the release of DNA due to autolysis serves as a raw material to drive transformation and evolution of bacterial population and minimize the accumulation of damages DNA or mutations (Redfield, 1988). However, loss of bacterial strains due to autolysis makes the bacterial strain a difficult experimental material (Pillich et al., 1974).
Although autoplaque provides an ecological and evolutionary advantage to the bacteria, the phenomenon has been mostly investigated in laboratory isolates having clinical importance. Degraded ecosystems are characterized by low density and diversity of microbes, and diverse abiotic and biotic stresses. Autoplaque affects the population density on the one hand but provides nutrients, secondary metabolites, and free DNA to support the growth and evolution of other members of populations on the other hand. Despite the ecological significance of autoplaque in the microbial community, not even a single study has been carried out on field isolates specifically from the degraded ecosystems. Analyzing the prevalence of autoplaque in spatially distributed microbial communities from degraded lands would be useful to improve microbial technologies for environmental restoration. Therefore, the present study investigated the prevalence of autoplaque phenomenon in bacteria associated with different soils particle size fractions of the spatially heterogeneous environment of degraded mine. The study reports the microbial community of degraded mine possesses a substantial level of autoplaque phenomenon, which has an association with variations in the elemental composition of different soil particle size fractions.

**Material and Methods**

**Study site and sample collection**

The Bhati mine was abandoned more than 100 years ago, which is characterized by highly heterogenous topography (undulating terrain with deep gullies, flat land to the slope, and pits of different sizes). The mine is located on the southern spur of Aravalli mountains in National Capital Territory of Delhi (28°24.2’N–77°13.6’E), India (Fig. 1). The area is primarily denuded with patchy vegetation comprising of weedy ephemerals. The goethite red sand type of soil of Bhati mine is was characterized by insignificant levels of nutrients and organic matter (Sharma et al., 2011). Delhi shows varying level of annual precipitation (~150 mm to ~1000 mm) and temperature (-2 to 48°C). The soil was sampled from three sites from an abandoned mine separated at a distance of 10m. The soil samples were collected using acid-treated polypropylene digger in black polyethylene and transported to the laboratory (Fig. 6). The soil was immediately used for elemental analyses (Pachauri et al., 2013) and purification of bacterial communities (Wommack et al., 2009; Koskella et al., 2011; Sharma et al., 2011).

**Soil fractionation**

The soil was fractionated using the standard method outlined by Kabir et al., (1994). One hundred gram soil was taken in 250ml Erlenmeyer flask containing sterile distilled water and kept at 4°C for 36h. After incubation, the soil suspension was passed through a set of sieves arranged from top to bottom in decreasing order of pore sizes, >2000µm, 2000-210µm and 210-63µm to separate gravel, coarse sand and fine sand, respectively. However, the coarse silt fraction (63-20µm) was settled by gravity, fine silt fraction (20-2µm) was pelleted down by low-speed centrifugation (90×g, three times) of the unsettled supernatant, and clay fraction (<2µm) was obtained by calcium chloride flocculation followed by centrifugation(2460×g for 5 min). All soil fractions were kept at 4°C for further experiments.

**Elemental analysis of soil particles**

The soil particles were characterized for the composition of micronutrients and heavy metals (Si, Co, Cu, Fe, Mn, Ni, Zn, Cr, Cd and Pb), and macronutrients (C, Ca, K, Mg, P, S) by scanning electron microscopy– energy
dispersive X-ray system (SEM-EDX; Oxford 6841) using standard protocol (Pachauri et al., 2013). The soil samples were coated with palladium using glow discharge method (SPI-Module Sputter Coater), and EDX spectra of each soil sample were analyzed for qualitative and quantitative variations in elemental composition.

Isolation of bacteria community

The bacterial communities comprising of taxonomically close members of Micrococcaceae were purified on selective media (von Rheinbaben and Hadlok, 1981). The soils collected from three sampling points were suspended separately in sterile distilled water (in triplicate) and plated on FP agar media [supplemented with 0.02% (w/v) Furazolidone] selective for Micrococcaceae. Bacterial colonies appeared till 48h were picked randomly, and purified by transfer of well-isolated colony on fresh media plate consecutively for five times. Three bacterial communities comprising of five hundred forty bacterial isolates were purified from four soil particle size fractions (Fig. 6). The glycerol stocks of all the 540 bacterial isolates were prepared in triplicate and stored at 80°C for further experiments (Koskella et al., 2011; Sharma et al., 2011).

Estimation of autoplaque phenomenon in bacterial community

The overnight bacterial cultures were raised in FP broth at 30°C in shaking condition (130 rpm). A 50µl of bacterial culture (~2×10^8 cells) was plated on FP agar media plate and kept in BOD incubator at 30°C for 48h. Spontaneous appearance of the clear zone was taken as positive for autoplaque phenomenon (AP+). A continuous layer of bacterial lawn formed after 48h of bacterial growth was taken as autoplaque negative (AP−) (Breyen and Dworkin, 1984). A total of 1320 autoplaque assays were conducted.

Statistical analysis

The difference in the frequency of autoplaque producing bacteria among three spatially distributed microbial communities (BC1, BC2, and BC3) was compared using Chi-square test. Similarly, a difference in the frequency of autoplaque producing bacteria of the microbial communities associated with different soil particle size fractions, i.e., gravel, sand, silt, and clay, were compared using Chi-square test. The difference in the elemental composition (micronutrients, heavy metals, and macronutrients) in soils/soil particle size fractions were compared using one-way ANOVA

Result and Discussion

The autoplaque phenomenon is one of the biological processes which serve as a major determinant of ecology and evolution of bacterial population and community. Primarily, autoplaquing has been investigated in clinically important bacterial isolates; however, the phenomenon has a high relevance in microbial communities of degraded ecosystems characterized by low density and diversity of microbes.

We selected the members of Micrococcaceae because they harbour various mechanisms to tolerate different inorganic and organic contaminants present in the degraded abandoned mine (Santhini et al., 2009). In fact, the Micrococcaceae members have also been used to remediate different contaminants. Therefore, we assessed the prevalence of autoplaque phenomenon among spatially distributed microbial communities and soil particle size fractions associated bacterial communities of taxonomically closed taxa (Micrococcaceae) purified from an abandoned mine. We showed that prevalence of autoplaque phenomenon significantly differs among bacterial communities associated with soil particle size
fractions as compared with the bacterial communities from spatially distributed soil (10m scale). The correlation between prevalence of autoplaque and elemental composition of soil particles (micro and macro elements, and heavy metals) was analyzed. The pattern of autoplaque distribution in bacterial communities showed an association with soil particles which significantly differ in elemental composition.

The autoplaque phenomenon has been observed in laboratory isolates after frequent sub-culturing or exposure of old cultures to high temperature (Sili et al., 2012) indicating its inducible nature. However, our study showed that a substantial proportion of bacterial isolates (~18.33%) of soil bacterial communities might show autoplaque even without frequent sub-culturing i.e. non-inducible autoplaque phenomenon. It further indicates that in contrast to the known effect of age of bacterial culture or stage of sub-culturing, the microbial community may also possess natural tendency to show autoplaque in culture conditions. In contrast with spatially distributed bacterial communities, the soil particle size fractions associated communities significantly differed in the incidence of autoplaque phenomenon. This is the first evidence on the prevalence of this phenomenon from the natural environment that too from Micrococcaceae members, which have not been shown to possess autoplaque phenomenon.

The occurrence of autoplaque in young bacterial culture and even without frequent sub-culturing suggests the ecological significance of autoplaque in bacterial communities of degraded ecosystems. It would be interesting to investigate the non-inducible and inducible autoplaque forming bacteria and identify the extrinsic and intrinsic factor(s) governing this phenomenon in bacterial community.

Different bacterial populations have been shown to have a very high level of autoplaque phenomenon (50–92%). In this context, our results appear to lie in a low range of autoplaque phenomenon. However, our study analysed on the microbial community of taxonomically closed taxa, whereas other studies were conducted on strains/isolates of a single species. In fact, 18.33% level of autoplaque at the community level may be considered a very high level of autoplaque phenomenon as it comprised of various strains, species and genera of Micrococcaceae rather than strains of single species. Also, most of the studies investigated population comprising of small to a moderate number of isolates in comparison with 540 isolates investigated in the present study. Further, a bacterial species also sustainably varies in the level and type of autoplaque depending on the culture conditions and environmental source of bacteria. For example, 90% of Pseudomonas arugensosa isolates (18 out of 20 isolates) showed autoplaque that too only after several sub-culturing (Berk, 1963). But the isolates failed to show autoplaque phenomenon without any sub-culturing. In another study, 90.66% of P. aeruginosa isolates showed autolysis when grown in specifically in tryptone–glucose agar medium (Berk, 1965). Similarly, 92% of P. aeruginosaisisolates (46 out of 50 isolates) showed spontaneous lysis and produced turbid, iridescent auto-plaques (AP+) (Berk and Gonkowski, 1964). Subsequently, Jones et al., (1974) demonstrated that 68.8% of P. aeruginosaisisolates (172 out of 250 isolates) show autoplaque. Fifty percent of isolates of Nodularia spumigena, a marine bacterium from the Baltic Seashowed 50% of autoplaque phenomenon driven by either cyanomyoviruses or cyanosiphoviruses (Silie et al., 2012). Therefore, to ascribe the level of autoplaque to bacteria species, we need to identify the isolates. Therefore, it requires a separate study.
**Fig. 1** The variations in the frequency of autoplaque formation in bacterial communities associated with soil particle size fractions (A); and spatially distributed soil (B)

![Graph](image1)

**Fig. 2** The variations in timing of appearance of autoplaque in bacterial communities associated with soil particle size fractions (A); and spatially distributed soil (B)

![Graph](image2)
Fig. 3 The variations in the proportion of bacterial isolates showing development of immunity (centred plaque) in bacterial communities associated with soil particle size fractions (A); and spatially distributed soil (B).
**Fig. 4** The variations in elemental composition of different soil particle size fractions (gravel, sand, silt and clay). Bars represent means ± SE of three replicates. Statistically significant differences at the 5% level are indicated by different letters over the appropriate bars and following a one-way ANOVA.
**Fig. 5** The variations in elemental composition of spatially distributed soil samples (at spatial scale of 10m). Bars represent means ± SE of three replicates. Statistically significant differences at the 5% level are indicated by different letters over the appropriate bars and following a one-way ANOVA.
Fig. 6 Scanning electron micrographs and energy-dispersive X-ray analysis of different soil particle size fractions i.e. gravel, sand, silt and clay, of the abandoned mine.
Table 1: Inferential statistics showing results of One Way ANOVA carried out to compare the content of micronutrients and heavy metals in the soil particles (gravel, sand silt, clay) and spatially separated sites.

| Element | Particle | Site |
|---------|----------|------|
| Pb      | F(3,32)=12.337 \(p=0.000\) | F(2,33)=0.242 \(p=0.787\) |
| Cd      | F(3,32)= 28.071 \(p=0.000\) | F(2,33)= 0.228 \(p=0.798\) |
| Ca      | F(3,32)= 239.553 \(p=0.000\) | F(2,33)= 0.1 \(p=0.905\) |
| Cr      | F(3,32)= 2.12 \(p=0.117\) | F(2,33)= 0.342 \(p=0.713\) |
| Mn      | F(3,32)= 0.453 \(p=0.717\) | F(2,33)= 1.311 \(p=0.283\) |
| Fe      | F(3,32)= 6.427 \(p=0.002\) | F(2,33)= 2.279 \(p=0.118\) |
| Co      | F(3,32)= 5.974 \(p=0.002\) | F(2,33)= 1.313 \(p=0.283\) |
| Ni      | F(3,32)= 8.037 \(p=0.000\) | F(2,33)= 1.322 \(p=0.28\) |
| Cu      | F(3,32)= 2.815 \(p=0.055\) | F(2,33)= 0.224 \(p=0.8\) |
| Zn      | F(3,32)= 5.63 \(p=0.003\) | F(2,33)= 1.22 \(p=0.308\) |
| K       | F(3,32)= 7.824 \(p=0.000\) | F(2,33)= 2.249 \(p=0.121\) |
| S       | F(3,32)= 27.191 \(p=0.000\) | F(2,33)= 0.579 \(p=0.566\) |
| P       | F(3,32)= 29.059 \(p=0.000\) | F(2,33)= 1.33 \(p=0.278\) |
| Mg      | F(3,32)= 20.445 \(p=0.000\) | F(2,33)= 2.19 \(p=0.128\) |
| C       | F(3,32)= 7.363 \(p=0.001\) | F(2,33)= 4.268 \(p=0.022\) |
| Si      | F(3,32)= 18.523 \(p=0.000\) | F(2,33)= 1.012 \(p=0.375\) |

The spatially distributed bacterial communities did not show significant difference in the extent of autoplaque phenomenon (chi-square \(\chi^2 (2)=1.360, \ p=0.523\), Fig. 1B). Further, variations in the levels of micro- and macro-elements and heavy metals of the spatially distributed soils were not significant (Fig. 5, Table 1). The spatially distributed bacterial communities did not show a significant difference in the timing of appearance of autoplaque (Fig. 2B). Similarly, some of the bacterial isolates showed the development of lysis-resistant mutant in the center of autoplaque. However, the proportion of bacterial isolates showing immunity towards lysis did not differ in spatially distributed bacterial communities (Fig. 3B).

In contrast with the spatially distributed bacterial communities, the soil particle size fractions significantly differed in the level of autoplaque phenomenon (chi-square \(\chi^2(9)=1.833, \ p=0.000\), Fig. 1A). The soil particle size fractions also showed a significant difference in the levels of C, P, K, Ca, S, Mg, Fe, Co, Zn, Ni, Si, Pb and Ca (Figs. 4 and 6, Table 1). The soil particle size fraction associated bacterial communities also differed in the timing of appearance of autoplaque. For example, ~80% of isolates from sand particle community showed a delay in autoplaque formation. However, >80% of isolates from silt particle showed early autoplaque formation (Fig. 2A). The level of immunity developed towards lysis (appearance of centered plaque) in the bacterial community showed a direct relationship with soil particle size fraction. For example, soil bacterial communities associated with high particle size fraction (gravel) showed a high level of immunity (Fig. 3A). As compared with the bacterial community associated with low particle size fraction (clay). Different studies have shown contradictory views on the role of nutrient and
environmental factors to drive autopleque phenomenon in bacteria. For examples, the classical studies from Berk (1963, 1965) suggested an influence of nutrient on autopleque production; however, Heurlier et al., (2005) suggested the lack of any effect of nutrients on autopleque production (Heurlier et al., 2005). However, Berk (1966) also showed that the self-lysis becomes more obvious when the bacteria are cultivated on complex growth media. Also, the synthetic chemicals (antibiotics, antiseptics, disinfectants) in low concentration (non-inhibitory levels) also increased the degree of lysis but a low concentration of antiseptic and disinfectant bacteriostatic retarded the rate and degree of lysis (Berk, 1965). Similarly, Breyen and Dworkin (1984) showed elevated temperature, and light condition failed to induce this phenomenon. On the contrary, Sili et al., (2012) showed a positive role of light and temperature coupled with the age of the culture on autopleque induction. The studies on the role of genotoxic chemicals showed a concentration dependent effect on autopleque phenomenon. For example, a single supplement antibiotic at non-inhibitory level increased the degree of lysis but a low level of antiseptic and disinfectant bacteriostatic reduced the rate and degree of lysis (Berk, 1965).

Both intrinsic and extrinsic factors may be responsible for autopleque formation. For example, in *P. aeruginosa*, the induction of pyocins or prophages and complex interaction between two different phages has been shown as a possible cause of autopleque phenomenon (Berk, 1963; Shaburova et al., 2001). Some studies showed that the frequency of the auto-plaque phenomenon in *Brucella* associated with the carrier state (pseudolysogeny) of the bacteria (Renoux and Suire, 1963). However, in *B. subtilis* the proteolytic enzyme, namely endolysins mediate cell wall lysis during sporulation, but the enzymes derived from bacteriophage lytic enzymes (Loessner et al., 1997). Therefore, further in-depth analyses on bacterial communities purified from different soil particle size fractions would be useful to reveal the factors operating in nature determining the autopleque phenomenon. Though phage community has not been investigated in the present study, the role of the phage-induced autopleque phenomenon cannot be ignored. We propose that seasonal fluctuation in the environmental and soil factors along with the level of autopleque phenomenon would be required to establish the role of external factors determining the autopleque phenomenon. Further, the dynamics of density and diversity of microbial communities will help in determining the ecological and evolutionary significance of autopleque in microbial communities.

The influence of autopleque phenomenon or self-lysis in bacteria on evolutionary potential of clinically important bacteria has been widely studied. However, it has ecological implications on microbial communities of degraded ecosystems as it serves as a selection pressure affecting bacterial population density and also provides a splash of nutrients, secondary metabolites and free DNA for microbial community. Our study provides evidence that microbial community of degraded ecosystems that comprised of taxonomically closed taxa show a high level (~18.33%) of autopleque phenomenon. Unlike other laboratory studies, our study demonstrates that bacterial communities possess autopleque phenomenon even without sub-culturing or the effect of age of the culture. In fact, to the best of our knowledge, this is the first report suggesting the occurrence of autoplques in members of *Micrococcaceae*. The spatially distributed bacterial communities (at 10m scale) do not show a significance difference in autopleque phenomenon.
phenomenon but soil particle size fraction (gravel, sand, silt, and clay) associated bacterial communities substantially differ in the proportion of autoplaque producing isolates. The bacterial communities also showed the identical trend for the population of bacterial isolate characterized based on the timing of appearance of autoplaque and potential to develop immunity (centered plaque). The level of nutrients, trace metals, and heavy metals also showed the similar pattern suggesting their association with the pattern of autoplaque phenomenon observed in the study. The in-depth analyses of these bacterial communities will help to understand the role of extrinsic (environmental) and intrinsic (physiological) factors affecting the autoplaque phenomenon in bacterial communities. Such a fundamental knowledge on the relevance of autoplaque phenomenon in microbial communities would be useful to improve the microbial technologies for environmental restoration of degraded ecosystems.

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