Trends in Life Sciences
An International Peer-reviewed Journal
www.sciencejournal.in

BIOFILM MEDIATED SEQUESTERING OF METAL IONS BY *KLEBSIELLA VARIICOLA* PRBC 14

Rina Rani Ray*
Postgraduate Department of Zoology, Bethune College, 181, Bidhan Sarani, Kolkata: 700 006, India.
E-mail: raypumicro@gmail.com

ABSTRACT
The extensive industrial usage of the heavy metals and subsequent release of effluents, without proper treatment in the environment contaminates the ecosystem and causes remarkable health problems. There is a large interest in microorganisms that can facilitate the separation and removal of the metal contaminant. A metal tolerant strain *Klebsiella variicola* was isolated from the top soil dust of iron mine and was allowed to grow in presence of high amount of aluminum, manganese, nickel and stannous salts. The trend of slime production by the bacteria was studied photomicrographically and the biofilm production in presence and absence of metal ions were quantified. Gradual reduction in turbidity of the high concentration of metal supplemented medium after bacterial growth was measured. The bacterium was found to be a profuse producer of slime and slime production increased in presence of various metal ions. The efficacy of the strain to grow in presence of high amount of metal ions might be attributed to the biofilm that could trap to separate the metal ions and allowed the bacteria to thrive. Highest separation of Mn^{2+} , Al^{3+},Sn^{2+} and Ni^{2+} was found when the strain was grown in culture media supplemented with 6800mg/L,3150mg/L, 2960 mg/L, 4480 mg/L of respective metal ions. The strain adopted an intrinsic strategy to thrive in metal intoxicated environment and probably is correlated with its efficacy of biofilm production.

K**EYWORDS:** biofilm, *Klebsiella variicola*, metal tolerance, slime production.

INTRODUCTION

Heavy metals causing persistent environmental pollutants are usually introduced into the environment through anthropogenic activities resulting mainly from industrial effluents (Teitzel and Parsek, 2003). Bacteria play a pivotal role in the maintenance of the environmental homeostasis and have developed a variety of resistance mechanisms to counteract heavy metal stress. These mechanisms include the formation and sequestration of heavy metals in complexes, reduction of a metal to a less toxic species, and direct efflux of a metal out of the cell (Outten *et al*, 2000). The trapping of heavy metals may be accomplished through formation of biofilms.

Biofilms are cell-cell or solid surface-attached assemblages of microbes that are entrenched in a hydrated, self-produced matrix (Karatan and Watnick, 2009.) Bacteria growing in biofilms exhibit increased resistance to metals and antimicrobials (Chhibber *et al*,2013).Biofilm producing bacteria are usually embedded in an extracellular polymeric substance (EPS) matrix composed of polysaccharides, proteins, and nucleic acids (Vu *et al*, 2009), that provides increased resistance to antimicrobial agents compared to the resistance of free-swimming organisms (Hentzer *et al*, 2001). EPS components of the biofilm bind with the metal ions by negatively charged phosphate, sulfate, and carboxylic acid groups (Hentzer *et al*, 2001). These bound metals are then subsequently carried to the microbial cell walls where they are either taken inside of the cells and assimilated into non-toxic organic compounds or transformed from insoluble toxicant to soluble intoxicant form (Hunt, 1986).

It was found that stationary-phase cells were more resistant to a variety of different antimicrobial agents (Spoering and Lewis, 2001) than the logarithmic phase cells and this resistance can be primarily attributed to the stationary phase or slow growth. Although the tolerance of bacterial biofilms to metal ions has been documented in a number of studies (Teitzel & Parsek, 2003; Harrison *et al*., 2004), the main focus was slime production by pathogenic strains obtained from various clinical or pathological centres. It was proposed that biofilms are capable of removing heavy metal ions from bulk liquid (Huang *et al*, 2000 and Labrenz *et al*, 2000), and hence can be used to remove heavy metals from wastewater (White and Gadd, 1998, Chevallier *et al*, 1988). But for that a nonpathogenic strain may be more suitable to use.

The present study deals with the production of biofilm and trapping and sequestering of some metal ions by the biofilm produced by a nonpathogenic strain of *Klebsiella variicola* isolated from abundant iron mine area.

MATERIALS AND METHODS

Collection of soil sample

Soil samples were collected from the top soil of Khonbond iron mine (at 21°57 min 18.02 sec North and 85°23 min 15.4 sec East) located at an altitude of 676 meter in Keonjhar district of Odisha, India and were taken in sterilized polyethylene bags using sterilized spatula and stored at 4ºC until examination.

Isolation of the bacterial strain

The bacterial strain was isolated by cultivating in basal medium (BM) composed of (g/L): peptone, 0.9; $(NH_4)_2HPO_4$, 0.4; KCl, 0.1; $MgSO_4 \cdot 7H_2O$, 0.1 (pH-8.0).and 0.1 glucose at for 24-36 hours.

Identification of the bacterial strain

The bacterial strain was characterized by routine tests, for identification and was genetically identified using 16srRNA. It was later submitted to NCIM, Pune, India for confirmation.

Photomicrographic study

The crystal violet-saffranine stained bacterial cells were visualized under Axioscop-40 (Zeiss) microscope at 1009.For SEM, paraformaldehyde–glutaraldehyde fixed and totally dehydrated specimens were sputter coated with gold palladium under vacuum and observed and photographed in a scanning electron microscope (FEI Quanta-200 MK 2).

Quantification of biofilm formation

The bacterium was grown in normal culture medium for 0-60 hours. Each flask containing growth medium of different hour was diluted (1:100) with fresh medium. The flat bottom tissue culture plates (96 wells) were filled with 200µl of each type of diluted cultures individually. The culture plates were incubated at 37°C for 24 hours. After incubation, gentle tapping of the plates was done. The wells were washed with 200 µl of phosphate buffer saline (pH 7.2) four times to remove free-floating bacteria. Biofilms which remained adherent to the walls and the bottoms of the wells were fixed with 2% sodium acetate and stained with 0.1% crystal violet. Excess stain was washed with deionized water and plates were dried properly. Optical densities (OD) of stained adherent biofilm were obtained with a UV visible spectrophotometer (Shimadzu, Japan) at wavelength of 540 nm. The OD values of non inoculated sterile medium were taken as control. (Mathur *et al*, 2006). The data obtained were used to classify the strains as high producers (OD higher than 0.500), producers (OD between 0.500 and 0.100) or poor producers (OD lower than 0.100) (Maldonado *et al*, 2007).

Determination of tolerance to various metal ions

Strains were grown on Petri plates on solid basal medium supplemented with different metal ions namely, Mn^{2+}, Sn^{2+}, Ni^{2+} and Al^{3+} of different concentrations. A non inoculated plate without inoculums for each metal ion was maintained as control.

Measurement of turbidity

The turbidity of the culture was measured at 660 nm in a UV visible spectrophotometer (Shimadzu, Japan).

Estimation of separation of metallic ions

The bacterial strain was grown in presence of various concentrations of metal ions namely Mn^{2+}, Sn^{2+}, Ni^{2+} and Al^{3+} in liquid cultivation condition in 100 ml Erlenmeyer flask. The reduction of turbidity, caused by the suspended metal ions after 48 hours of growth was measured to estimate the change in the concentration of suspended metal ions. The viability of the bacterial cells after the growth in metal supplemented media was checked by sub culturing them on normal basal medium containing Petri plates.

All experiments were done in triplicate and their values were averaged.

RESULTS AND DISCUSSION

The isolated bacterial strain, identified as *Klebsiella variicola* showed a tendency to form a cell–cell attachment when grown in normal culture under optimal conditions (pH 8 at 28°C). As it moved towards log phase of growth, it changed its phenotypical profile to become an active slime producer and the slime became gradually denser (Fig 1). The present

strain at early to mid-stationary phase of growth was found to be a profuse producer of slime as evident from the SEM study (Fig 2). Probably this ability of biofilm production allowed the bacterial strain to thrive under environmental stress like metal toxicity, as according to Gaidhani *et al*, 2014, biofilm is an important mode of bacterial life fostering elevated resistance towards antibiotics and metal salts. The optical densities (OD) of stained adherent biofilm produced by the present strain indicated that slime production achieved its highest level at mid stationery phase of growth (Fig 3) and the present working strain could produce a profuse amount of slime (Fig 3) as the change in optic density at 540 nm was found to be greater than 0.5 **(**Maldonado *et al*, 2007).

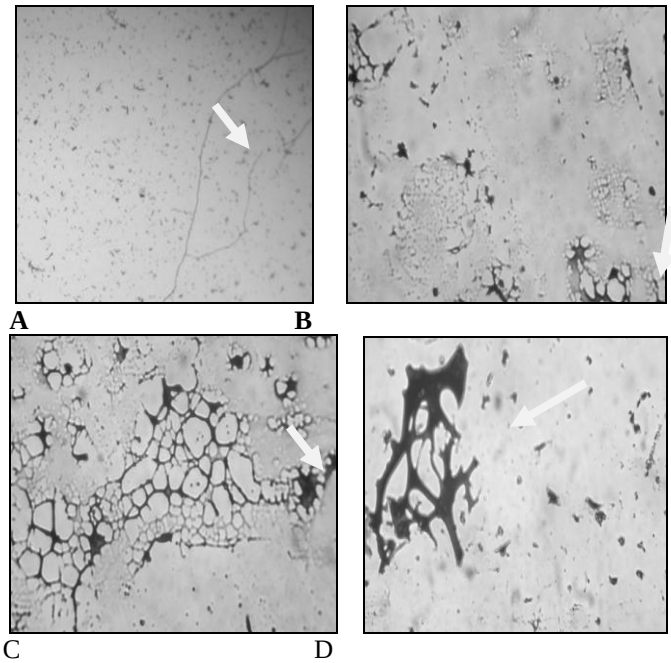

Fig 1 Trends in biofilm production by normally growing *Klebsiella variicola* PRBC 14, Growth at: **A**: 12 hrs, **B**: 24hrs, **C:** 48 hrs, **D:** 72 hrs

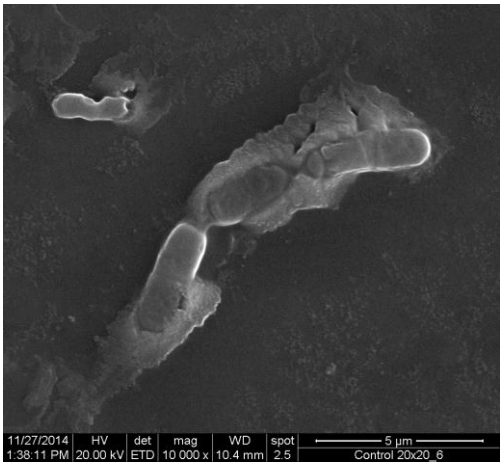

Fig 2. Scanning electron micrograph of *Klebsiella variicola* PRBC 14 producing slime at 40th hour of growth.

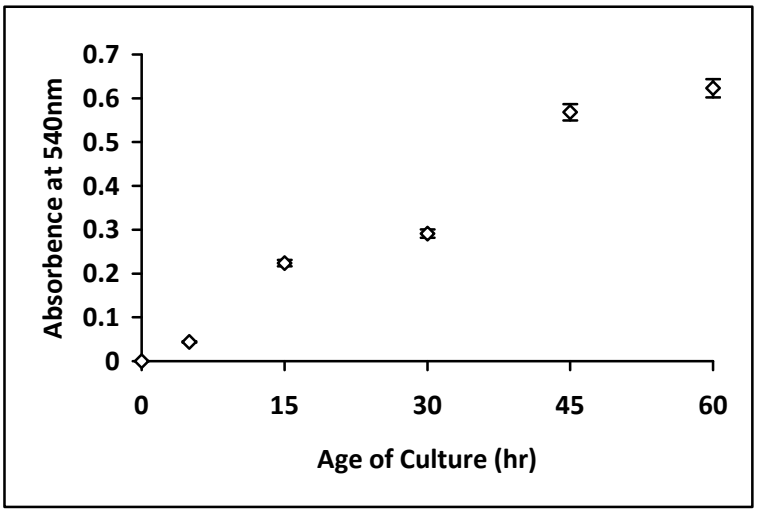

Fig 3. Kinetics of biofilm production by *Klebsiella variicola* PRBC 14

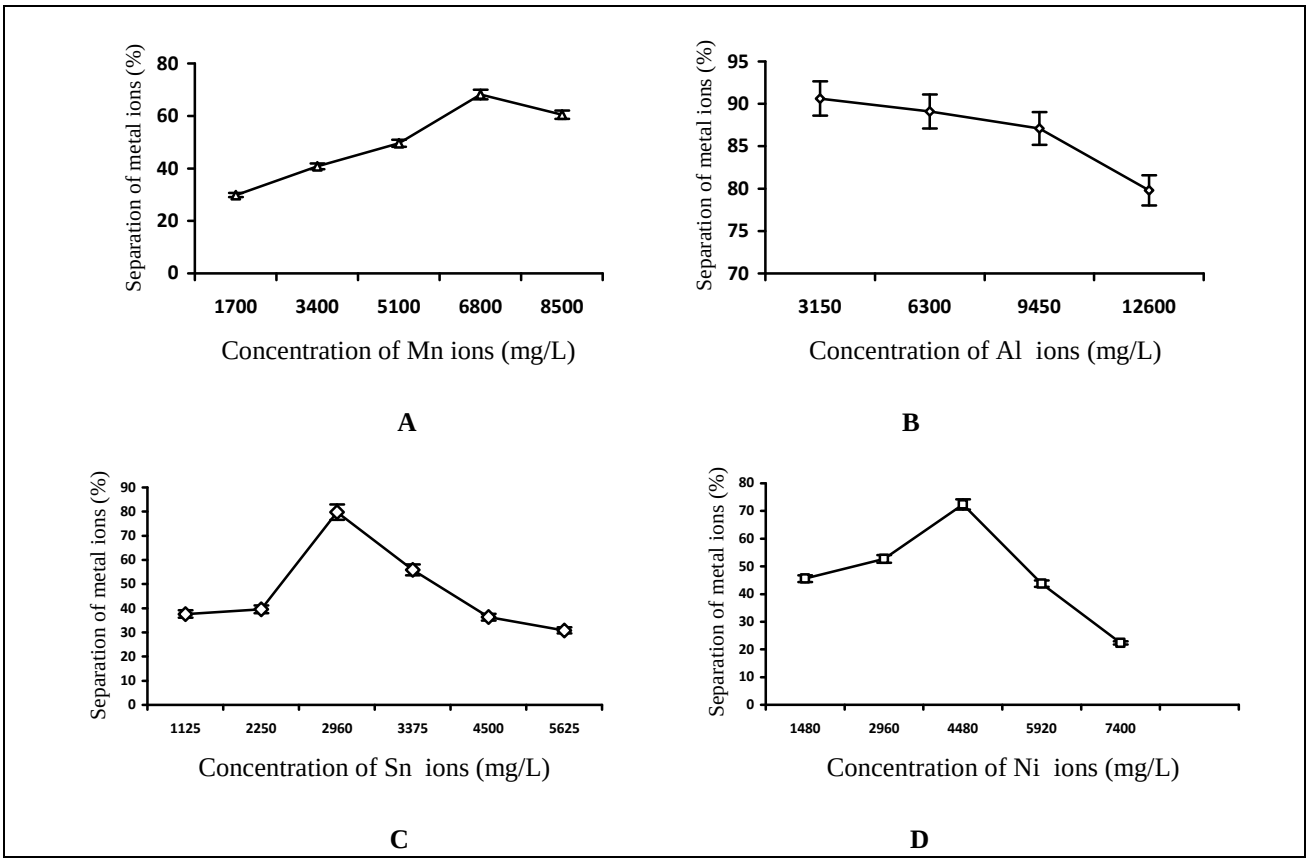

Fig 4. Seqestering of metal ions **A**: Mn^{2+}, **B**: Al^{3+}, **C**: Sn^{2+} and **D:** Ni^{2+} by *Klebsiella variicola* PRBC 14 as depicted by the percentage of changes of absorbance at 660 nm.

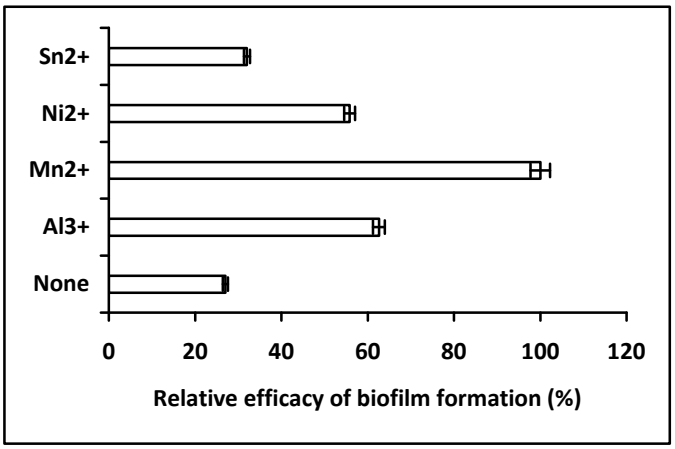

Fig 5. Biofilm formation in presence of heavy metals by *Klebsiella variicola* PRBC 14

The bacteria could grow on solid plate in culture supplemented with various toxic metal ions like Mn^{2+}, $Al^{3+,}$ Sn^{2+}, Ni^{2+}. The rate of bacterial growth was most rapid in presence of Sn^{2+} and Ni^{2+} followed by $Al^{3+.}$ The feeblest growth was found in case of Mn^{2+} supplemented medium. In liquid cultures, the bacterial strain was found to tolerate a higher concentration of metal ions than their solid counterparts (data not shown). In liquid medium, presence of metal ions in high concentration increased the turbidity of the medium due to suspension of the particles. But, with the gradual propagation of bacteria, amazingly the turbidity of the culture medium decreased, which was probably accomplished by the separation of metal ions from the medium. Appearance of bacterial colonies, after sub culturing on solid plate from the heavy metal intoxicated culture medium indicated the decrease in turbidity did not result from the death of the bacteria but could be interpreted as the consequence of trapping of these metal ions in the biofilm, produced by the bacteria. This phenomenon of sequestering of metal ions was also reported by Harrison et al., 2005 and it was found that biofilms are capable of removing heavy metal ions from bulk liquid (Huang *et al***,** 2000).

In the present strain, this separation was found to be dependent on the type and concentration of metal ions. Highest separation of Mn^{2+} , $Al^{3+,}$$Sn^{2+}$, Ni^{2+} was found when the strain was grown in culture media supplemented with 6800mg/L,3150mg/L, 2960 mg/L, 4480 mg/L of respective metal ions (Fig 4), above which the separation of ions reduced, probably due to the limited capacity of the biofilm to trap the ions. The exopolymeric substances (EPS) , composed of charged polymers such as polypeptides, nucleic acids and polysaccharides make EPS act as ionic resin, to check tthe entry of metal ions into the interiors of biofilm (Teitzel *et al*, 2003). Hence, with the course of biofilm formation, the growth rate of bacteria was found to be decreased, although a few cells could survive as "persistor cell" in presence of high concentration of metal ions (Drenkard, 2003). Biofilm formation through slime production was considered as an intrinsic strategy through which microbes might survive exposures to antimicrobial agents. Since, the change of absorbance at 660 nm was related to total metal ions and slime level of the sample, its overall reduction clearly indicated the trapping of metal ions by biofilm produced by the bacteria. This view was contrary to the findings where *E. coli, Klebsiella sp. and Pseudomonas aeuroginosa* were found to lose their ability to form biofilm at higher concentrations of some of the metals (Goel *et al*, 2011). In the present strain, the correlation between metal resistance and biofilm formation was confirmed by the relative efficacy of the strain to produce slime in presence of various metal ions (Fig 5). Highest production of biofilm was found in presence of Mn^{2+} followed by Al^{3+}, Ni^{2+} and Sn^{2+}.

CONCLUSION

From these results, this study concluded that the increase of heavy metal resistance was regulated by biofilm and the metal ions could be sequestered from surrounding medium through sorption on the EPS matrix. The strain with an ecological background of metal enriched soil of mine area, more precisely the tailings, the waste products generated during the recovery of the minerals must have an intrinsic strategy to thrive in metal intoxicated environment and probably its efficacy of biofilm production helped the strain to trap the toxic particles and provide it a safe shelter for its survival.

REFERENCES

Chhibber S., Nag D. and Bansal S. (2013). Inhibiting biofilm formation by Klebsiella pneumoniae B5055 using an iron antagonizing molecule and a bacteriophage. *BMC Microbiol.* 13:174 http://www.biomedcentral.com/1471-2180/13/174

Drenkard E. (2003). Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms. *Microb. Infect.* 5: 1213-1219. http://www.ncbi.nlm.nih.gov/pubmed/14623017

Gaidhani S.V., Raskar A.V., Poddar S., Gosavi S., Sahu P.K., Pardesi K.R., Bhide S.V. and Chopade B.A. (2014). Time dependent enhanced resistance against antibiotics & metal salts by planktonic & biofilm form of *Acinetobacter haemolyticus* MMC 8 clinical isolate. *Indian. J. Med. Res.* 140: 665-671.http://www.ncbi.nlm.nih.gov/pubmed/25579150

Goel G., Kaushik S., Beniwal V., Sandhu S.S. (2011). Effect of heavy metals on growth and biofilm formation by gram negative pathogens. *Current Trends in Biotechnol. Chem. Res.* 1: 102-105. http://ctbcr.com/index.php/biochem/article/view/23

Harrison J.J., Ceri H., Stremick C. and Turner R.J. *(*2004). Biofilm susceptibility to metal toxicity. *Environ **Microbiol. 6*: 1220-1227. http://www.ncbi.nlm.nih.gov/pubmed/15560820**

Harrison J.J., Turner R. J. and Ceri H. (2005). Persister cells, the biofilm matrix and tolerance to metal cations in biofilm and planktonic *Pseudomonas aeruginosa. Environ Microbiol.* 7: 981-994. http://www.ncbi.nlm.nih.gov/pubmed/15946294

Hentzer M., Teitzel G.M., Balzer G.J., Heydorn A., Molin S., Givskov M. and Parsek M.R. (2001). Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. *J. Bacteriol.* 183:5395–5401. http://jb.asm.org/content/183/18/5395.abstract

Hunt S. (1986). Diversity of biopolymer structure and its potential for ion binding applications, In H. Eccles and S. Hunt (ed), Immobilisation of ions by bio-sorption, Ellis Horwood Ltd, West Sussex, United Kingdom. Pp 15–46. https://books.google.co.in/books?isbn=0203483049

Huang Y.B., Wang W.H. and Peng A.(2000). Accumulation of Cu(II) and Pb(II) by biofilms grown on particulate in aquatic systems. *J. Environ. Sci. Health, Part A: Toxic/Hazardous Subs. Environ. Engineering* 35: 575–592. http://www.tandfonline.com/doi/abs/10.1080/10934520009376987

Karatan E. and Watnick P. (2009). Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiol.Mol.Biol.Rev*. 5:310–347. www.ncbi.nlm.nih.gov/pubmed/19487730

Labrenz M., Druschel G.K., Thomsen-Ebert T., Gilbert B., Welch S.A., Kemner K.M., Logan G.A., Summons R.E., De Stasio G., Bond P.L., Lai B., Kelly S.D. and Banfield J.F. (2000). Formation of sphalerite (ZnS)deposits in natural biofilms of sulfate-reducing bacteria. *Sci.* 290:1744–1747. **http://www.sciencemag.org/content/290/5497/1744.abstract**

Le Chevallier M.W., Cawthon C.D. and Lee R.G. (1988). Factors promoting survival of bacteria in chlorinated water supplies. *Appl. Environ. Microbiol.* 54: 649–654. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC202520/

Maldonado N.C., Silva de Ruiz C., Cecilia M., Nader-Macias M.E. (2007). A simple technique to detect *Klebsiella* biofilm-forming-strains, inhibitory potential of *Lactobacillus fermentum* CRL 1058 whole cells and products. In : A. Méndez-Vilas (ed), Communicating Current Research and Educational Topics and Trends in Applied Microbiology, FORMATEX,Spain,52-59. http://www.formatex.org/microbio/pdf/Pages52-59.pdf

Mathur T., Singhal S., Khan S., Upadhyay D.J., Fatma T.and Rattan A. (2006). Detection of biofilm formation among the clinical isolates of Staphylococci: An evaluation of three different screening methods. *Indian. J. Med. Microbiol.* 24(1): 25-29. http://www.ijmm.org/text.asp?2006/24/1/25/19890

Outten F.W., Outten C.E. and O'Halloran T. (2000). Metalloregulatory systems at the interface between bacterial metal homeostasis and resistance, In: G. Storz and R. Hengge-Aronis (ed), Bacterial stress responses.ASM Press, Washington, D.C.Pp145–157. http://www.scholars.northwestern.edu/bookChapterDetail.asp?id=998&o_id=195

Spoering A.L. and Lewis K. (2001). Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J. Bact.* 183:6746–6751. http://www.ncbi.nlm.nih.gov/pubmed/11698361

Teitzel G.M. and Parsek M.R. (2003). Heavy Metal Resistance of Biofilm and Planktonic *Pseudomonas aeruginosa*. *Appl.Environ.Microbiol.* 69: 2313–2320. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC154819/

Vu B., Chen M., Crawford R.J.and Ivanova E.P. (2009). Bacterial Extracellular Polysaccharides Involved in Biofilm Formation. *Molecules.* 14: 2535-2554. http://www.mdpi.com/1420-3049/14/7/2535

White C. and Gadd G.M. (1998). Accumulation and effects of cadmium on sulphate-reducing bacterial biofilms. *Microbiol.* 144:1407–1415. http://mic.sgmjournals.org/content/144/5/1407.

