Chapter 2

Characterization of putative autolysin mediated eDNA release and biofilm formation

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### Chapter 2: Characterization of putative autolysin mediated eDNA...

#### Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>88</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>89-90</td>
</tr>
<tr>
<td>2.2 Material and methods</td>
<td>90-94</td>
</tr>
<tr>
<td>2.2.1 Bacterial strains and culture conditions</td>
<td>90</td>
</tr>
<tr>
<td>2.2.2 DNA and RNA manipulations</td>
<td>90</td>
</tr>
<tr>
<td>2.2.3 Gene identification, amplification and DNA sequencing</td>
<td>90</td>
</tr>
<tr>
<td>2.2.4 Transcriptional analysis by RT-PCR</td>
<td>91</td>
</tr>
<tr>
<td>2.2.5 Cloning-expression and analysis of proteins by SDS-PAGE</td>
<td>91</td>
</tr>
<tr>
<td>2.2.6 Analysis of LysM and MltB protein in silico</td>
<td>92</td>
</tr>
<tr>
<td>2.2.7 Phenotypic Characterization of clones</td>
<td>92</td>
</tr>
<tr>
<td>2.2.7.1 Peptidoglycan binding assay</td>
<td>92</td>
</tr>
<tr>
<td>2.2.7.2 Autolysis Assay</td>
<td>92</td>
</tr>
<tr>
<td>2.2.7.3 Total Viable Count</td>
<td>93</td>
</tr>
<tr>
<td>2.2.7.4 Cell surface hydrophobicity (HI Index)</td>
<td>93</td>
</tr>
<tr>
<td>2.2.7.5 eDNA release</td>
<td>93</td>
</tr>
<tr>
<td>2.2.7.6 Biofilm augmentation</td>
<td>93</td>
</tr>
<tr>
<td>2.2.8 Statistical Analysis</td>
<td>93</td>
</tr>
<tr>
<td>2.3 Results</td>
<td>94-99</td>
</tr>
<tr>
<td>2.3.1 PCR and RT-PCR results</td>
<td>94</td>
</tr>
<tr>
<td>2.3.2 Cloning and expression in E. coli DH5a</td>
<td>95</td>
</tr>
<tr>
<td>2.3.3 Bioinformatics analysis of LysM and MltB proteins</td>
<td>96</td>
</tr>
<tr>
<td>2.3.4 Phenotypic characterization of the clones</td>
<td>96</td>
</tr>
<tr>
<td>2.3.4.1 Peptidoglycan binding ability</td>
<td>96</td>
</tr>
<tr>
<td>2.3.4.2 Autolysis</td>
<td>97</td>
</tr>
<tr>
<td>2.3.4.3 Total cell viability</td>
<td>98</td>
</tr>
<tr>
<td>2.3.4.4 Increased hydrophobicity</td>
<td>98</td>
</tr>
<tr>
<td>2.3.4.5 Enhanced eDNA release</td>
<td>99</td>
</tr>
<tr>
<td>2.3.4.6 Biofilm augmentation</td>
<td>99</td>
</tr>
<tr>
<td>2.4 Discussion</td>
<td>100-102</td>
</tr>
<tr>
<td>2.5 References</td>
<td>103-105</td>
</tr>
</tbody>
</table>
Abstract:
The earlier chapter described contribution of extracellular DNA (eDNA) to biofilm formation in clinical strain *Acinetobacter baumannii* AIIMS 7. In this chapter, investigation was furthered to evaluate autolysis mediated release of eDNA and to understand the possible underlying mechanism. Two genes, encoding lysin binding domain (*lysM*) and murein lytic transglycosylase (*mltB*) were identified in this study. Cloning and over-expression showed 23 kDa and 37 kDa proteins encoded by the genes. Bioinformatics analysis indicated the presence of a conserved motif of 40 residues in LysM (specific to peptidoglycan-binding activity); whereas MltB was found to contain a transglycosylase-like motif of 144 residues specific for catalytic cleavage of N-acetyl muramic acid and N-acetyl glucosamine residues present in murein of cell wall. A series of phenotypic characterization and assessment of heterologously over-expressed *lysM* and *mltB* clones were performed; which showed significant peptidoglycan binding ability, autolysis and lowering in total viable count. Furthermore, enhanced eDNA production, increased hydrophobicity index (HI) and biofilm augmentation on polystyrene was observed. The results collectively indicated that the eDNA resulting from autolysis can facilitate biofilm formation on abiotic surface. It is concluded that LysM and MltB proteins could be associated with autolysis at early growth phase of *A. baumannii* AIIMS 7; and thereby constitute a mechanistic basis of eDNA facilitated-biofilm formation in this pathogen.

**Keywords:** *Acinetobacter baumannii*, extracellular DNA (eDNA), lysin binding domain (*lysM*), murein lytic transglycosylase (*mltB*), autolysis, biofilm formation.
Chapter 2: Characterization of putative autolysin mediated eDNA...

2.1 Introduction:

*Acinetobacter baumannii* is involved in a wide range of infections in hospitals and has posed a major challenge to current antibiotic era due to multi-drug resistance (MDR) (Perez *et al*., 2007, Peleg *et al*., 2008). Pathogenesis of severe infections by *A. baumannii* is poorly understood (Gordon and Wareham, 2010). Biofilm formation is a major virulence factor in clinical isolates of *A. baumannii* (de Breij *et al*., 2010; Lee *et al*., 2008; Loefhelm *et al*., 2008). Biofilm formation has been aptly described as a multi-factorial regulatory process in *A. baumannii* (Antunnes *et al*., 2011, Gaddy and Actis, 2009). *A. baumannii* is capable of forming biofilms on abiotic and as well biotic surfaces and often associated with infection (Tomaras *et al*., 2003; Lee *et al*., 2008; Loefhelm *et al*., 2008; Pour *et al*., 2011); and a certain degree of variation in biofilm formation is shown to be dependent on the strain type as well (McQueary and Actis, 2011). Nevertheless, association of specific determinants with formation and fine-tune regulation of *A. baumannii* biofilm at molecular level is still to be portrayed fully. Therefore, the current understanding on specific mechanisms operating behind biofilm development needs to be magnified. This, in a long run would certainly help in broadening the scope for molecular microbiologists and drug-designers; to devise specific molecular target-based strategies to combat biofilm associated infections by MDR *A. baumannii*; one of the major concerns in healthcare units worldwide (Cerquiera and Peleg 2011, Gordon and Wareham, 2010).

Extracellular DNA (eDNA) plays the role of a critical determinant in biofilm development by its scaffolding nature and can be inhibited by the action of DNase (Whitchurch *et al*., 2002). It is also known as a key component of bacterial biofilm matrix (Flemming and Wingender 2010; Chiang and Tolker-Nielsen, 2010). It originates from the intracellular DNA which is independent of cell-lysis at early phases of growth (Hara and Ueda, 1981). Release of eDNA by autolysis enhances biofilm adhesion and surface aggregation and strengthening (Whitchurch, 2002; Das *et al*., 2010). Role of eDNA in biofilm development and the probable underlying mechanisms has been understood in *Staphylococcus aureus*, *Enterococcus fecalis*, *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* (Rice *et al*., 2007; Qin *et al*., 2007; Heilmann *et al*., 1997; Ma *et al*., 1999; Allessen-Holm *et al*., 2006).

Role of eDNA, its characterization and release in MDR strain *A. baumannii* AIIMS 7 was demonstrated for the first time in chapter - 1 (Sahu *et al*., 2012). The study showed that the clinical strain *A. baumannii* AIIMS 7 released eDNA into the extracellular milieu and augmented biofilm biomass to substantial amounts. Further, eDNA was characterized and found to be in free form as well as encapsulated inside membrane vesicles of various sizes 20...
Chapter 2: Characterization of putative autolysin mediated eDNA...

- 200 nm. Importantly, the eDNA was shown to have a profound role in biofilm augmentation on abiotic surface. To investigate further, the mechanistic basis of the eDNA release which is independent of natural cell death and membrane vesicles, it was hypothesized that the release of eDNA could be via action(s) of putative autolysins. In the present study, two putative genes were identified, encoding for lysin binding domain (\textit{lysM}) and murein lytic transglycosylase (\textit{mltB}) respectively in \textit{A. baumannii} AIIMS 7. Further investigation was carried out for characterization of these two putative autolysin-like proteins and to evaluate their association with autolysis-mediated eDNA release and facilitation of biofilm formation.

2.2 Material & Methods :

2.2.1 Bacterial strains and culture :

The multidrug resistant clinical strain \textit{A. baumannii} strain AIIMS 7, used in the previous chapter was used in this study. \textit{E. coli} DH5\(\alpha\) was used for all cloning and expression experiments and recombinant \textit{E. coli} cultures were maintained on LB broth and agar plates supplemented with 100 \(\mu\)g / ml ampicillin as and when required.

2.2.2 DNA and RNA manipulations :

\textit{A. baumannii} AIIMS 7 genomic DNA and (Sigma Aldrich, USA). Recombinant plasmids were purified using a Plasmid Miniprep kit (Sigma Aldrich, USA). DNA, RNA and plasmid samples were quantified in Biophotometer plus (Eppendorf, Germany). Samples were also analyzed by agarose gel electrophoresis.

2.2.3 Gene identification, amplification and DNA sequencing :

Using reference genome map of \textit{A. baumannii} AB0057, two sets of primers were designed and used to amplify two regions referred to as lysin binding domain (\textit{lysM}) and murein lytic transglycosylase (\textit{mltB}) using \textit{A. baumannii} AIIMS 7 genomic DNA as PCR template. All the primers are listed in Table 2.1. The primers were designed using tool Primer Quest (Integrated DNA Technologies, USA) and primer BLAST program (NCBI, USA); and synthesized from sigma Aldrich (USA). Gradient PCR programs were used to optimize the annealing temperatures and specific amplification. After specific amplification, both the genes (amplicons) were eluted from gel using a Gel Extraction Kit (Bangalore GeNei, India). To rule out any chance of plasmid borne putative autolysins, PCR amplification was attempted using native plasmids of \textit{A. baumannii} AIIMS 7. All PCR chemicals, enzymes and primers were purchased from Sigma Aldrich. Nuclease free water (Bangalore GeNei, India) was used as negative PCR control. Purified PCR products were subjected to DNA sequencing in a
3730 DNA Analyzer (Applied Biosystems, USA) using methods as described in Chapter-1. Nucleotide sequences for obtained for *lysM* and *mltB* genes, were deposited to Genbank (NCBI, USA).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer F/R</th>
<th>Sequence (5’-&gt;3’)</th>
<th>Length</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>lysM</em></td>
<td>lysM-F</td>
<td>CGCAACTAACTTTAGATCCCCAAGCC</td>
<td>26</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>lysM-R</td>
<td>AGGGCACAACATTGCTTCTGGAATGA</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td><em>mltB</em></td>
<td>mltB-F</td>
<td>GTGTCCCTTACCTTTATGAGT</td>
<td>23</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>mltB-R</td>
<td>TGTGTGTATTGTGAAGCGATTTAAC</td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>

### 2.2.4 Transcriptional analysis by RT-PCR:
Total RNA was purified by using Genei TRI solution (Bangalore GeNei, India), as per manufacturer’s instructions. Total RNA was quantified using BioPhotometer (Eppendorf, Germany). The isolated RNA was treated with DNase I to rule out any presence of genomic DNA in the sample. The first strand synthesis was performed by heating 10 µl RNA template and 3.7 µl nuclease free water at 65°C for 10 min, followed by incubating the mixture at room temperature for 2 min. To this reaction mix, 1 µl random hexamer, 1 µl MMuLV reverse transcriptase, 0.3 µl Human placental RNasin, 1 x enzyme buffer and 2 µl dNTP were added and incubated at 37°C for 1 h and then at 95°C for 2 min to inactivate reverse transcriptase. The c-DNA was then further used for PCR amplification of *lysM* and *mltB* and the products were analyzed by agarose gel electrophoresis.

### 2.2.5 Cloning-expression and analysis of proteins by SDS-PAGE:
The purified PCR products of *lysM* and *mltB* were appended with 5’-A overhangs using a final extension of 72°C for 15 min in the respective PCR programs of both genes. These products were then ligated to TA cloning and expression vector system pGEMT-Easy (Promega, USA) using T4 DNA ligase and as per manufacturer’s instructions. The ligated recombinant plasmids were then transformed in to chemically competent *E. coli* DH5α using methods described (Sambrook and Maniatis 1987). The *lysM* and *mltB* clones were obtained on ampicillin (100µg/ml) containing LA plates and were confirmed using colony PCR. The check the heterologous expression of the LysM domain protein and MltB
protein in \textit{E. coli} DH5α, whole cell extracts of the clones were analyzed by and SDS-PAGE (12.5% gels) followed by staining with Coomassie Brilliant Blue.

\textbf{2.2.6 Analysis of LysM and MltB protein \textit{in silico}:}

In order to predict the probable molecular mass of LysM and MltB proteins, structure and different features of the proteins, \textit{in silico} methods were used. The bioinformatics tools from the ExPASy Proteomics server (http://expasy.org/) like Translate Tool (http://expasy.org/tools/dna.html) and ProtParam (http://expasy.org/tools/protparam.html) were used for the primary and secondary structure analysis of the protein. Swiss-MODEL (http://swissmodel.expasy.org/) was used for building the 3-d model of the proteins LysM and MltB.

\textbf{2.2.7 Phenotypic Characterization of clones:}

\textbf{2.2.7.1 Peptidoglycan binding assay:}

Peptidoglycan (PG) binding ability of the heterologously expressed \textit{lysM} and \textit{mltB} clones was evaluated using methods (Heffron \textit{et al.}, 2011) with suitable modifications. Commercially available peptidoglycan (HiMedia, India) was used as the substrate for testing the binding efficacy of the two aytolysin-like proteins LysM and MltB. 200 µl each of the cell free supernatants collected from 9 h old grown \textit{lysM} and \textit{mltB} cultures, were respectively incubated along with PG (2mg/ml) at 25°C in a final volume of 1 ml of 30 mM sodium phosphate buffer (pH 7.0) consisting of 1 mM EDTA, 1 mM DTT, and 0.1 % (v/v) Triton X-100. LB broth was used as the negative control in which PG was supposedly unable to bind. Purified lysozyme (5 mg/ml) was used as positive control presuming effective binding with PG. After starting the reaction, OD600 was recorded at every 2 min interval until 30 min in a Multi-plate Reader (Molecular Devices, USA). The experiment was repeated on three different instances.

\textbf{2.2.7.2 Autolysis Assay:}

The autolysis assay was performed as described by Del Papa \textit{et al.}, (2007) with few modifications. The overnight grown culture of clones and control cells, with O.D. adjusted (OD 600 = 0.8), were again sub-cultured by adding 3 % (w/v) glycine to the medium and incubated overnight at 37°C. The cells were obtained by centrifuging at 13,000 rpm for 3 min followed by washing thrice with ice-cold sterile distilled water and suspended in 10 mM sodium - phosphate buffer (pH 6.8). The autolysis profile was analyzed by measuring the absorbance at 600 nm and plotting the values on to Excel spreadsheets.
2.2.7.3 Total Viable Count

In order to monitor the cell lysis in *lysM*, *mltB* clones and host *E. coli DH5α*, total viable count was taken. For determination of total viable count (TVC), overnight grown culture was serially diluted in physiological saline and plated in duplicates on LA plates followed by incubation at 37°C for 24 h. The TVC was calculated in terms of CFU/ml.

2.2.7.4 Cell surface hydrophobicity (HI Index):

The hydrophobicity assay was performed as described by (Rosenberg and Gutnick 1980). The cell pellet of 1.5 ml of 24 h grown culture of the clones and control cells was washed with PUM buffer (K₂HPO₄: 22.2 g/L, KH₂PO₄: 7.26 g/L, Urea: 1.8, MgSO₄·7 H₂O: 0.2 g/L, pH 7.4). The initial absorbance of the suspended pellet was measured at 540 nm (A₀), followed by addition of 0.14 ml *p*-xylene and vortexing for 15 min. After phase separation for 30 min, the absorbance of aqueous phase was measured at 540 nm (A₁). The hydrophobicity index was calculated as: HI = 100 - (100 × A₁ / A₀).

2.2.7.5 eDNA release:

To evaluate eDNA release by clones with respect to control host, purification of eDNA from the culture of the clones and controls was carried out as per methods described in chapter 1 (Sahu et.al., 2012). The eDNA was suspended 20 µl DNase-free TE buffer provided by Sigma Aldrich and quantified in a Biophotometer Plus (Eppendorf, Germany).

2.2.7.6 Biofilm augmentation assay:

Quantitative biofilm augmentation was assessed using modified microtitre plate method (Lee et.al., 2008) using *lysM/mltB* clones; control *E. coli DH5α* and *A. baumannii* AIIMS 7 strains.

2.2.8 Statistical Analysis

Data from the experiments were entered in to excel spreadsheets (Microsoft, USA). Mean and standard deviations were estimated from three independent replicate experiments. Statistical significance of the results was tested using Student’s two tailed t-test, P value < 0.05 was considered to be statistically significant.
2.3 Results:

2.3.1 PCR and RT-PCR results:
The amplification \textit{lysM} by PCR and RT-PCR assays can be seen below (Fig. 2.1-A). Similarly, amplification of \textit{mltB} by PCR and RT-PCR assay is demonstrated in Fig 2.1-B. The GenBank accession numbers obtained for the two genes were JF914076 and JN205072 respectively for \textit{lysM} gene (cds:614 bp) and \textit{mltB} gene (amplicon size : 1061 bp, cds: 999 bp). The features of the sequences can be found in the GenBank flatfiles (Appendix).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{Fig_2.1-A_Amplification_of_lysM_by_PCR_and_RT-PCR.jpg}
\caption{Fig. 2.1-A Amplification of \textit{lysM} by PCR and RT-PCR}
\begin{itemize}
\item Lane 1, 5: Negative PCR and RT-PCR control,
\item Lane 2,4: PCR and RT-PCR amplification for \textit{lysM},
\item Lane 3: DNA ladder (100 bp - 3 kb)
\end{itemize}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{Fig_2.1-B_Amplification_of_mltB_by_PCR_and_RT-PCR.jpg}
\caption{Fig. 2.1-B Amplification of \textit{mltB} by PCR and RT-PCR}
\begin{itemize}
\item Lane 1, 5: PCR and RT-PCR amplification for \textit{mltB} gene,
\item Lane 2,4: Negative PCR and RT-PCR control for \textit{mltB},
\item Lane 3: DNA ladder (300 bp - 3 kb)
\end{itemize}
\end{figure}
2.3.2 Cloning and expression in *E. coli* DH5α

The transformed *E. coli* DH5α cells containing lysM and mltB genes respectively were analyzed for the protein product by SDS-PAGE. It can be seen that lysM gene encoded a distinct protein product close to 23 kDa (Fig. 2.2-A) whereas the mltB gene showed a protein band of ~37 kDa (Fig. 2.2-B) on SDS-PAGE, which were not expressed by the control *E. coli* DH5α containing the empty vector in both experiments.

![Image](image-url)

**Fig. 2.2-A: Analysis of cloned LysM protein (23kDa) on SDS-PAGE**
Lane 1: Total protein profile of *E. coli* with empty vector,
Lane 2: Total protein profile of *E. coli* with LysM band (arrow)
Lane 3: Standard protein molecular weight marker (14-94 kDa)

![Image](image-url)

**Fig. 2.2-B: Analysis of cloned MltB protein (36.8 kDa) on SDS-PAGE**
Lane 1: Total protein profile of *E. coli* with empty vector,
Lane 2: Total protein profile of *E. coli* with MltB band (arrow),
Lane 3: Standard protein molecular weight marker (14-94 kDa)
2.3.3 Bioinformatics analysis of LysM and MltB proteins:

The results from ExPASy server showed the theoretical molecular mass of LysM and MltB proteins are 23.06 kDa and 36.8 kDa respectively. Further analysis indicated the presence of 40 residue long conserved motif (74 - 117), (THKVKKGENLSLISHRFNCKISEIVHLN-KLKNPDHIDVGQII-KIP) also known as ‘Lysine Motif’ a small domain involved in binding with peptidoglycan. The Swiss Model generated 3-D model of LysM Lysine Motif (Fig 2.3A). MltB protein sequence had two regions, a ‘Transglycosylase SLT domain’ (34 to 327) which contained Lytic Transglycosylase (LT) and Goose Egg White Lysozyme (GEWL) domain (161-250) of 144 residues; (DELAALIAWTYKEGYPTNSIV-GSYAGAIGFPQFMPNISKYGVDFGNGHIDLRNDAEDAIGSIANYLAKQGWQR-DQPIGFMARYTGSN) which is known to cleave N-acetylmuramic acid and N-acetylg glucosamine residues in cell wall peptidoglycan. The binding site residues (173, 194, 227, 245) of MltB protein were found specific for binding of N-acetyl-D-glucosamine. 3-D model of MltB can be seen below (Fig. 2.3 B).

Fig 2.3 (A-B) 3-D Model of the LysM (A) and MltB (B) proteins as computed by Swiss-MODEL

2.3.4. Phenotypic characterization of the clones:

2.3.4.1 Peptidoglycan binding ability:

Observations of the assay for evaluating the clones for peptidoglycan binding (Fig. 2.4) explains that lysM clones do exhibit a better ability of binding with the cell wall component as compared to mltB clones wherein % initial OD 600 was reduced by 28 % compared to 22 % (p < 0.05). Lysozyme represented a positive control and the LB broth serves as negative control indicating no binding with the Peptidoglycan (no change in % initial OD).
2.3.4.2 Autolysis profile:

Comparative autolysis pattern during active growth (till 8 h) for the clones and control cells are demonstrated in Fig. 2.5. The graph shows a distinction between the control (with empty vector) cells and the clones containing \textit{lysM} and \textit{mltB} genes. However, it was seen that cells expressing LysM protein exhibited a steady pattern, almost equivalent to the control cells. In case of \textit{mltB} clones the autolysis was considerable in which the initial O.D. decreased up to 30%. This suggests that MltB protein may have a higher efficacy of autolysis than LysM within 8 h of growth.
2.3.4.3 Total cell viability:

Total viable count was estimated in order to observe the possible effect of the cloned product (LysM and MltB proteins) on the cells and overall effect in autolysis. The results suggest significant reduction of TVC in MltB clones as compared to that of its host (control cell with empty vector). For higher dilutions, the count was so low that it could not be represented in the graph. This observation was indicative that autolysis could be triggered by the putative autolysin - like proteins (MltB and LysM) due to which the cell count dropped drastically as compared to control cells.

![Fig.2.6. Total cell viability of the clones and control cells](image)

2.3.4.4 Increased hydrophobicity:

Hydrophobic nature of microorganisms enhances the chances of attachment and adherence to various surfaces. An increase in hydrophobicity index of the clones therefore indicates the possibility of enhanced biofilm formation. Hydrophobicity index of A. baumannii AIIMS 7 (47 %) is much higher compared to the control E. coli DH5α (p <0.05) (Figure 2.7). In comparison to the control LysM clones exhibited insignificant increase in HI (4 %, p >0.05) and mltB clones exhibited significant increase (22 %) in HI; suggesting that the MltB protein may be associated with the increased cell surface hydrophobicity which may support adherence and biofilm formation.
2.3.4.5 Enhanced eDNA release:

The study was intended at finding autolysis – mediated eDNA release and association of autolysin like proteins with effective cell lysis. The results demonstrated that in comparison to the control cells, clones exhibited enhanced eDNA release (133.5% and 212.26% for lysM and MltB respectively; p<0.04). eDNA production was two times more than MltB clones. This indicated MltB protein has better ability of releasing eDNA triggered by autolysis during active growth compared to LysM protein.

2.3.4.6 Biofilm augmentation assay:

Biofilm formation ability of the clones and control cells on abiotic surface (polystyrene microtitre) strongly indicated that both the clones lysM and mltB have significant ability to form biofilm in comparison to control cells (p<0.02, maximum 160.15% increase in mltB
clone). LysM clones showed biofilm augmentation of 142.49% compared to control cells. This allows us to assume that the event of autolysis might have resulted in the overall eDNA amount and finally contributing to the biofilm matrix which is observed to be augmented here.

\[
\begin{array}{c|c|c|c|c}
 & \text{AIIMS 7} & \text{E. coli control} & \text{lysM} & \text{mltB} \\
\hline
\text{Biofilm index (OD570)} & 4 & 3.5 & 3 & 4 \\
\end{array}
\]

\textbf{Fig. 2.9 Biofilm augmentation assay on polystyrene surface}

2.4 Discussion:

Formation of complex bacterial communities or biofilm, is a process that is of multifactorial nature, complex and highly regulated at the genetic level (Antunes et al., 2011; Gaddy and Actis, 2009). It is by now established that eDNA has a profound role in biofilm formation (Whitchurch et al., 2002) via its contribution to the biofilm matrix. eDNA can be useful to the matrix in multiple manners and therefore can influence the overall make up of the biofilm formed by the bacteria. There have been several studies using various bacterial systems, to explore the importance of eDNA mediated biofilm formation and the most accepted theory was autolysin - mediated eDNA release and thereby enrichment of biofilm. This however, remained an elusive area in case of the MDR pathogen \textit{A. baumannii}. In chapter - 1, characterization of eDNA was carried out as one of the important determinant of biofilm formation; therefore it was necessary to explore the underlying mechanism behind the eDNA release. The present chapter deals with the investigation of eDNA mediated DNA release via action of putative autolysins. The autolysin - like genes \textit{lysM} and \textit{mltB} were identified from the genome of \textit{A. baumannii} AIIMS 7 and characterized further to reveal their association with autolysis and subsequent biofilm formation via eDNA release.

Earlier studies, for example, in \textit{S. epidermidis}, eDNA has been shown to be essential in the initial phase of biofilm development on glass and polystyrene surfaces (Qin et al., 2007). In the same report, role of autolysin AtlE in release of eDNA and its function in biofilm has
been demonstrated. Interestingly, it was suggested that the eDNA was made available at the cost of killing a sub-population of the bacteria and the remaining population utilizing the eDNA for biofilm formation. Protease gelatinase (GelE) is the autolysin of *E. faecalis* which plays a significant role in *E. faecalis* biofilms and up-regulated in response to the quorum sensing molecule gelatinase biosynthesis activating pheromone (Thomas *et al.*, 2009). In comparison with these studies, this study is the first report of any autolysin in *A. baumannii* which proposes the action of these two putative autolysins LysM and MltB to cause autolysis (Fig. 2.4, 2.5), and release the DNA for biofilm formation, which was seen in the results (Fig.2.8 and 2.9). This is why this investigation hold a key role in the *Acinetobacter* biofilm-research; which can be furthered to examine a complete picture of autolysin-mediated biofilm formation *via* a speculated fratricidal mechanism of eDNA release, similar to that of *S. epidermidis* (Qin *et al.*, 2007). Subsequently, suitable inhibitors could be designed to control biofilm associated infections by *A. baumannii*, which are persistent and difficult to treat with the currently available antibiotic therapies.

Several bacteria and bacteriophages produce a variety of enzymes that cleave peptidoglycan either to lyse host cells or to re-model the cell wall during growth or cell division. *Bacillus subtilis* has been reported to have probable and definite 35 peptidoglycan hydrolase genes which are clustered into 11 families (Kunst *et al.*, 1997). Each of these families has a distinct characteristic and varied members. The families comprise of LytC amidase, lytD glucosaminidase, DL-endopeptidase I and II, LD - endopeptidase, lysostaphin, enterococcal muramidase, LrgB, XylA amidase and germination - specific lytic enzymes (Smith *et al.*, 2000). These different classes of peptidoglycan degrading enzymes cleave the molecule at different points. Certain enzymes like muramidases and lysozymes, cleave at the glycosidic linkages of the glycan strand. Amidases cleave the amide bond between N-acetyl muramic acid and the L alanine that forms part of the peptide cross-links, and peptidases cut amide bonds in the peptide cross-links. Many of these enzymes are modular and are composed of catalytic units linked to a number of other domains. Every enzyme consists of a characteristic catalytic N-terminal domain that is fused to C - terminal region containing multiple repeats, approximately of 40 residues in *Enterococcus hira* (Joris *et al.*, 1992). In accordance with this, the present study results from *in silico* protein analysis show that LysM contain a Lysine domain of 40 residues in length and is found in variety of enzymes involved in bacterial cell degradation. The function of the domain is to bind to the peptidoglycan. Presence of a conserved motif of 40 residues in LysM indicated peptidoglycan-binding activity.
In *E. coli*, a membrane-bound lytic murein transglycosylase D (MltD) consisted of an N-terminal transglycosylase domain with two LysM repeats at the C terminus (Bateman and Bycroft, 2000). Although there was no evaluation of the function of the domain, but using phylogenetic distribution, it was predicted that it was involved in flagellar biosynthesis. In contrast to this study, the present study evaluated the association of MltB protein with cell wall degradation and involvement in autolysis mediated eDNA release and biofilm formation. The *in silico* analysis of MltB sequence supported the same, showing the presence of transglycosylase-like motif of 144 residues specific for catalytic cleavage of N-acetyl muramic acid and N-acetyl glucosamine residues present in murein of cell wall.

The experimental evaluation of these predicted functions (from *in silico* analysis) confirmed the hypothesis that these autolysins, mostly that MltB is able to bind with PG (Fig. 2.4) of the cell and thereby cause autolysis which was seen in the autolysis assay (Fig. 2.5) and reduction in total viable counts (Fig. 2.6) during active stages of normal growth. Besides this, the overall characterization of the clones presented a picture of involvement of the two autolysins in increase in hydrophobicity, enhanced eDNA release and biofilm augmentation. It was also significant to observe that amongst the two, it’s the MltB which exhibited a higher efficacy of PG binding activity, autolysis, and eDNA release and therefore a net increase in the biofilm formation. However, a detailed characterization of *lysM* and *mltB* genes using a library of knock-out mutants would provide a thorough understanding of the functional association and the factors affecting their optimal activities. Also it would be interesting to study the interaction of these to proteins, especially LysM, being a lysine binding domain.

In summary, this chapter provides an interesting insight in to the process of biofilm formation in *A. baumannii* via action of two newly identified autolysin-like proteins lysM and MltB. Through phenotypic assessment of the heterologously expressed *lysM* and *mltB* clones, it was found that these are associated with peptidoglycan binding ability, autolysis, lowering in total viable count, increased hydrophobicity, enhanced eDNA release, and finally substantial augmentation of biofilms on polystyrene. It is concluded therefore, that the putative autolysins LysM and MltB proteins identified and characterized in *A. baumannii* AIIMS 7; could be associated with autolysis of a subpopulation at early growth phases; and thereby may constitute a mechanistic basis of autolysis mediated eDNA release and biofilm formation. Further investigation on the structure and functions of these putative autolysins may help in designing inhibitors to control biofilm formation in the pathogen *A. baumannii*. 
2.5 References:


