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Preparation of Bacterial Ghosts for *E. coli* JM109 Using "Sponge-like Reduced Protocol"

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ABSTRACT

It is important to use a simple protocol for Bacterial Ghosts (BGs) preparation. Recently, a new protocol has been introduced based on simple chemical compounds to prepare the BGs. The protocol has been given the name "Sponge-like" (SL). The used microbial strain was *E. coli* BL21 (CDE3) pLysS (Promega). Experimental Design (ED) has been used to optimize the preparation conditions. In general, to map the best conditions for BGs preparation, several steps have been followed and lead to a long protocol. There is a need for reducing the protocol steps. In this study and based on that strains within the same species are similar and aiming to introduce a simple BGs protocol, another *E. coli* recombinant strain, the *E. coli* JM109 (Promega) has been used. The optimum conditions obtained from the SL protocol were used. However, the MIC and the Minimum Growth Concentration (MGC) were determined. Light and electron microscope as well as the protein and the DNA contents were used to evaluate the BGs quality (BGQ). This study establishes a simpler protocol given the name "Sponge Like Reduced Protocol "SLRP". The protocol proves to be suitable for BGs preparation from strains within the same species and could be used for native or recombinant antigens preparation.

Key words: Bacterial Ghosts, reduced protocol, *E. coli* JM109

INTRODUCTION

Bacterial Ghosts is an important tool for preparing bacterial envelopes either for the use as drug delivery system or stimulating the immune system (Furst-Ladani *et al*., 1999; Jalava *et al*., 2002; Lubitz *et al*., 2009; Muhammad *et al*., 2012; Amara *et al*., 2013). The *E. lysis* gene which is a part of the bacteriophage genome (phage ΦX174) is used for bacterial ghost preparation. The importance of the *E. lysis* gene has been observed because it is the step of the phage release from *E. coli* (Hutchison and Sinsheimer, 1966). DNA nuclease is used to degrade any existed genetic materials could code for pathogenic proteins (Haidinger *et al*., 2003). BGs have been used in a range of applications. As an examples, they are an effective delivery system particularly for the native and recombinant antigens (Kang and Curtiss III, 2003; Amara *et al*., 2013). The unique

criteria govern the BGs quality is that the cells contain no DNA, proteins and cytoplasmic constituents (Amara *et al*., 2013). Recently, Amara *et al*. (2013) have described a protocol to monitor the DNA and the Protein content release during each step of the BGs preparation. Some chemical compounds which are able to generate BGs have been used in this protocol instead of the *E. lysis* gene. The concentrations of the different used chemical compounds were adjusted to their minimum amounts which are enough to insure the remove of the DNA and the Protein as well as the cytoplasm constituents with a successful 3D bacterial cell wall structure (BGs). In the SL protocol, the Plackett Burman design has been used to optimize the "Sponge like" protocol (Plackett and Burman, 1946). Empty bacteria or BGs will insure that the bacteria will not be able to replicate or transform any harmful constituents particularly those could affect or interact with the genomic DNA upon their use in any formula given to the human. However, BGs are a packages ready, to be refilled with any components and could be used as a delivery system, or even so the mother cells' wall could be loaded with new antigen using the molecular biology tools (when the cells still live) (Ebensen *et al*., 2004; Jalava *et al*., 2003; Kudela *et al*., 2005; Paukner *et al*., 2004, 2005). Or use as it is to get the benefit from the BGs 3D structure with the native cell wall antigenic sites which able to activate the immune system. This functionless bacteria cells still have their antigenic structure on their surfaces (Felnerova *et al*., 2004; Amara *et al*., 2013). This study is aimed to reduce the steps of the "Sponge-like" protocol described by Amara *et al*. (2013), particularly because of the use of similar *E. coli* recombinant strain (JM109) for the BGs preparation. This will prove the validity of the "Sponge-Like" protocol in the BGs preparation from *E. coli* JM109, is to be used in molecular biology experiments and protocols (Kiljunen *et al*., 2005; Li *et al*., 2002; Lee *et al*., 1990; Jalava *et al*., 2002). This will facilitate the research for loading antigens by genetic engineering tools in such recombinant strains or using them as a safe drug delivery system.

METHODS

Bacterial strain: *E. coli* recombinant cells JM109 (Promega) was used in this study. The *E. coli* genotype is: EndA1, recA1, gyrA96, thi, hsdR17 (rk-, mk+), relA1, supE44, Δ(lac-proAB), [F', traD36, proAB, laqI q ZΔM15]

Bacterial cultivation: The *E. coli* JM109 has been cultivated in 500 mL NB (in one liter flask) under static condition at 37°C for 72 h.

Determination of the MIC and MGC for NaOH, SDS and H_2O_2: The MIC and MGC for NaOH, SDS and H_2O_2 were determined for each using standard criteria (Andrews, 2001). $CaCO_3$ was used in two concentrations, 1.05 and 0.35 μg mL^{-1} as described by Amara *et al*. (2013).

Sponge-like reduced protocol for preparing the BGs: Two experiments with different parameters (Table 1) were conducted. The two experiments were selected from the results obtained by Amara *et al*. (2013) for the best conditions for BGs preparation. They are mainly experiments number one and eleven in the SL protocol.

Only the combination type of the experiments variables (either +1, or -1), while the real value of the +1 and -1 have been used from the determined concentrations of both of MIC and MGC. MIC for each of the used variables is the +1 variable values and MGC for each of the used variables is the -1 variable's value. The used steps were at the same as that described in SL protocol. The only difference was found in the MIC and MGC of the SDS. For more details refer to Amara *et al*. (2013).

Table 1: Experiment one and two for BGs preparation

	Experiment variables					Basic experiment		H_2O_2 step		Ethanol step		
Experiment No.	NaOH	$CaCO_3$	H_2O_2	SDS	Shacking-Temperature	Protein μg mL^{-1}	DNA μg mL^{-1}	Protein μg mL^{-1}	DNA μg mL^{-1}	Protein μg mL^{-1}	DNA μg mL^{-1}	BGQ (%)
1	-1	1	1	1	1	2589.255	160.0	190.710	12.35	63.081	2.75	100
2	-1	-1	-1	1	1	4223.493	88.5	384.354	27.00	73.350	6.00	100

JM109 BGs evaluation using light microscope: Bacterial smear for the both preparations were stained using crystal violet. The cells were examined using the light microscope. The cells' quality was determined for each preparation based on the quality of the 3D structure as either being correct or deformed. The overall BGQ for each was given as a (%).

Determination of the DNA and protein concentration: The concentration of the DNA and the protein were derived from the spectrophotometer measurement as described by Amara *et al.* (2013).

Sample preparation for electron microscope examination: Electron microscope was used to evaluate the bacterial cells 3D structure. The dry bacterial smear was coated with approximately 15 nm gold (SPI-Module Sputter Coater).

Scanning of the BGs surface: The golden coated sample then were scanned using analytical scanning electron microscope (Jeal JSM-6360LA). The secondary element was at 10 kV acceleration voltage (at room temperature). The digital images of the samples then, were adjusted and saved for further investigation.

***E. coli* JM109 viability for the BGs preparation:** The BG preparations were evaluated for the existence of any still viable cells. A sample is taken from each preparation and grow in NA plats. The plates then were incubated (in the incubator) at 37°C for five days (Amara *et al.*, 2013).

Agarose gel electrophoresis: Agarose gel electrophoresis was used to evaluate the DNA. The method has been conducted as described by Amara *et al.* (2013).

RESULTS AND DISCUSSION

This protocol is a reduced form of the protocol recently described by Amara *et al.* (2013). It is different from the common used protocols for BGs preparation which depend mainly on using the *E.* lysis gene (Furst-Ladani *et al.*, 1999; Jalava *et al.*, 2002; Lubitz *et al.*, 2009). The protocol minimizes the steps which could lead to the preparation of the BGs.

Determination of MIC and MGC: The MIC and MGC of *E. coli* JM109 for each of the NaOH and H_2O_2 has been as same as those of *E. coli* BL21 (CDE3) pLsS (Promega) and represent 0.0138 N and 0.00231 N, (+1, -1) for NaOH and 40.8 and 5.83 μL mL^{-1} (+1, -1) from 30% H_2O_2 for the H_2O_2, respectively. In case of SDS the (+1, -1) values were 0.237 mg mL^{-1} and 0.033 mg mL^{-1} of SDS respectively. In case of $CaCO_3$ the used amount of +1 value was 1.05 μg mL^{-1} while -1 value was 0.35 μg mL^{-1}.

Determination of *E. coli* viability: None of the cells in each of the preparation upon cultivation on NA medium for five days at 37°C has showed any growth.

Evaluation of the Sponge-Like Reduced Protocol: The BGs preparation protocol using *E.* lysis gene has been established and used frequently. However, recently Amara *et al.* (2013) have recalled the classical chemical methods for preparing the BGs. As described by Amara *et al.* (2013), "SL" protocol is designed to use cheap and safe chemical compounds for BGs preparation. Each of NaOH, SDS, H_2O_2 and $CaCO_3$ was used. The MIC and MGC for each was determined and used as +1 or -1 value. The BGQ was determined by the aid of each of the light and electron microscope. The amounts of the DNA and protein released during each step were determined. The protocol concerns not only the BGs preparation but also detecting any still viable cells and their lysis. This protocol and the chemical compounds were used, could be introduced as a general protocol for BGs preparation from other microbial strains. Simple methods and cheap chemical compounds have been used. It is not logic to follow the many steps which have been described in the long protocol "Sponge like" (Amara *et al.*, 2013) each time if one goes to use strain from the same species (Particularly, the optimization experiments). For that, in this study, we have used only the conditions which have given the best results and which represent experiments no 1 and 11 in the original paper. To validate such protocol another *E. coli* strain, JM109 has been used. The main idea of the original protocol is to use different concentrations in random conditions. Those concentrations are MIC and MGC. One is responsible for the cell death and the other responsible for minimizing the killing effect of the chemicals used. While we have used another different *E. coli* strains (even from the same species), we have determined both of the MIC and MGC for each of NaOH, H_2O_2 and SDS. The results even similar, as expected but one compound has given different results. It was the SDS. JM109 is more sensitive to the SDS than BL21(CDE3). The original protocol has given the name of "Sponge-like" While the chemical compounds introduces a micropores in the bacterial cells. Upon centrifugation, during the BGs preparation, the cells have been pressed gently like the sponge to release their content without the deformation of its 3D structure which is the most important thing. H_2O_2 guarantee the degradation of any rest of the DNA. The experiments have been controlled by light microscope and by electron microscope. The cell quality proves to be same as that have been gained in the original protocol as shown in Fig. 1a and b. and in Table 1. The agarose gel results prove that the entire DNA in the final step has been completely degraded as in Fig. 2. The chemical compounds used in this study prove to be efficient

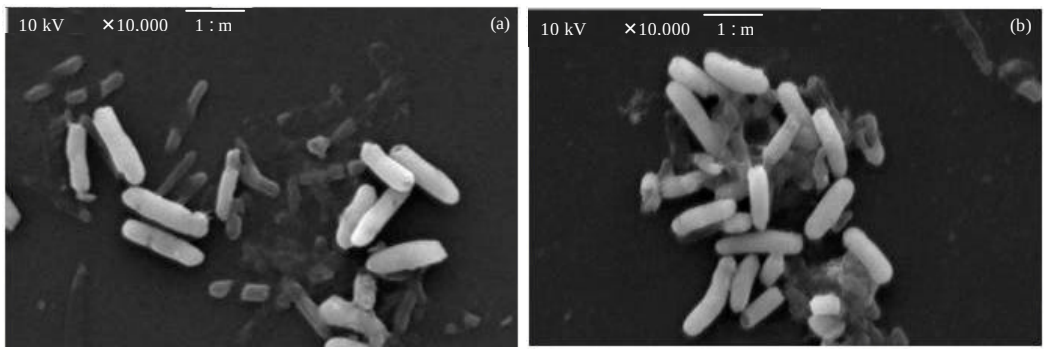

Fig. 1(a-b): Scanning electron microscope for *E. coli* BG cells. (a) *E. coli* JM109 from first experiment, (b) *E. coli* JM109 from second experiment

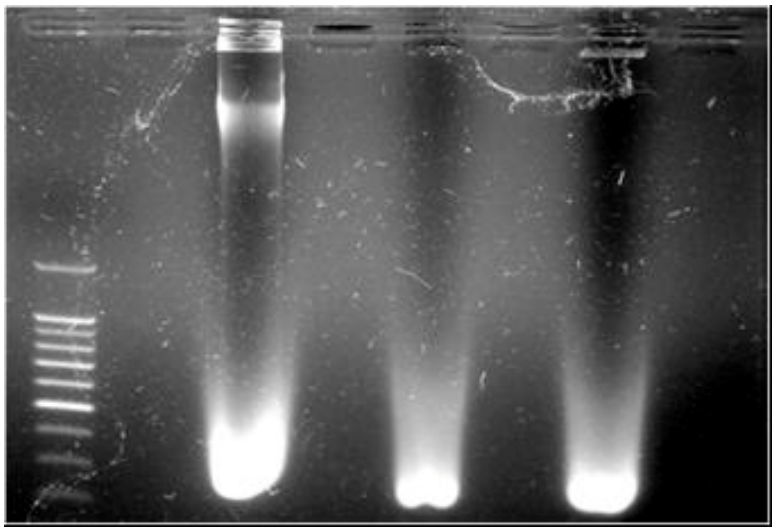

Fig. 2: AGE for the marker (first lane), control (second lane) and *E. coli* JM109 preparation from experiment one and two (third and fourth lanes)

if they have been used in the correct concentration to insure the successful BGs preparation. We should highlight that NaOH has been used in its -1 value in both experiments. SDS and "Shaking rate and Temperature" (used as one variable) are the same. The amount of the DNA in the first experiment upon the use of H_2O_2 (+1) is less than that in experiment 2 (-1) where they are 12.35 and 27 μg mL^{-1}, respectively as in Table 1. This is a logic result. Higher amount of H_2O_2 (+1) does more degradation to the DNA which upon quantification spectrophotometrically will give less reading. In Table 1 both experiments are same in the first step conditions except the $CaCO_3$. $CaCO_3$ has been used in its +1 value in experiment number one and its -1 value in experiment number two, apparently; somehow $CaCO_3$ might be responsible for increasing the DNA release and decreasing the protein release. It is clear that a minor change in the experimental parameters and conditions could influence the results. For that we recommended using precise steps to prepare the BGs using this protocol. Using the original protocol "SL" for preparing BGs from new species other than *E. coli* must be followed to fix any missed parameter, enable randomization and optimization, then using the optimum obtained results (Experiments) parameters and conditions to prepare the BGs from other strains in the same species. However MIC and the MGC for the new strain must be determined. We have been given this protocol the name "Sponge-Like Reduced Protocol" "SLRP" for BGs preparation. Determining the MIC and the MGC will polish the strain variations expected effect or error and will allow better BGs preparation.

CONCLUSION

In this study a previous introduced protocol for BGs preparation has been reduced. The optimum conditions of the original protocol have been only used. Another *E. coli* strain (JM109) has been used to validate the reduced protocol. While it is a different strain, its MIC and MGC have been determined. The reduced protocol has been successfully used to produce high quality BGs. The BGs quality has been controlled as described in the original protocol by determining its 3D structure quality using light and electron microscope. The released proteins and DNA during the BGs preparation have been determined spectrophotometrically. The complete

degradation of the bacterial genomic DNA has been determined using agarose gel electrophoresis. The reduced protocol succeeded to produce the BGs and has given the name "Sponge Like Reduced Protocol" "SLRP".

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