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A novel protocol for bacterial ghosts' preparation using tween 80

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ABSTRACT

Bacterial ghosts (BGs) can be prepared by both genetic and chemical means. Genetic method include using lysis gene *E*. Chemical method include incubation with numerous agents for a short time at their minimum inhibitory or minimum growth concentrations (MIC or MGC). The aim of this study is to prepare the BGs with a new protocol via exposing the bacterial cells to tween 80 for an extended period of time followed by sudden reduction of the surrounding pH. *Salmonella enterica serovar typhimurium* ATCC 13311 was used for this purpose. The cells were incubated in 7% $^{v}/_{v}$ tween 80 solution in Muller-Hinton broth for 24 h at 37 °C then pH was decreased to 3.6 by adding lactic acid for one hour. The bacterial pellets were separated by high speed centrifugation, and then washed three times by half normal saline solution. High quality BGs were visualized by scanning electron microscopy (SEM) revealing punctured cells with intact outer shells and at least one intramembranous tunnel. The absence of vital cells was confirmed by subculturing. The release of respective amounts of proteins and DNA is another evidence of ghost's production. In addition, the integrity of cells was proved by visualization of Gram-stained cells using light microscopy. In conclusion, this new protocol is simple, economic and feasible for BGs preparation.

1. Introduction

The need for safe vaccines, biological carriers and effective drug delivery systems has increased tremendously in recent times. The Bacterial Ghosts (BGs) are one of the biotechnology platforms that can satisfy this need. BG, by definition, is an empty cellular envelop of Gram-negative bacteria conserving their surface and antigenic characters and lacking the internal components (Langemann et al., 2010). This definition was confined to Gram-negative bacteria because of their unique ability to translate highly specific protein E of Phage ϕX174, which was the only means of creating BGs till recently. This protein can create transmembrane tunnel which leads to cell death without full lysis (Lubitz et al., 2009). Gene E of the Phage ϕX174 encode this 91-aa lysis protein (Kwon et al., 2005). Under controlled translation of this gene into the lysis protein has been used for production of Gram-negative bacterial (*Escherichia coli*, *Salmonella typhimurium*, *Salmonella enteritidis*, *Klebsiella pneumoniae*, *Bordetella bronchiseptica, Heliobacter pylori, Vibrio cholerae, Haemophilus influenza, Pasteurella multocida, Pseudomonas aeruginosa*) ghosts (Haidinger et al., 2003; Langemann et al., 2010). The definition of BG has been extended to include Gram-positive bacteria by virtue of using specific chemical agents in critical concentrations and critical times (Vinod et al., 2015).

Bacterial ghosts are useful in different biotechnology applications. Vaccine delivery system using ghost preparation offers effective humoral and cellular immune responses (Peng et al., 2011). Veterinary vaccines also can be prepared using the respective ghosts (Kwon et al., 2006). Targeting chemotherapeutic agents as doxorubicin to human colon cancer cell line was very effective utilizing genetically obtained *E. coli* ghosts leading to less side effects of the anticancer (Paukner et al., 2004). The effective internalization of *Escherichia coli* and *Mannheimia haemolytica* ghosts by human conjunctiva epithelial cells was used as beneficial drug

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carriers (Kudela et al., 2011). Additionally, BGs carrying DNA can be used in gene therapy (Tabrizi et al., 2004; Kudela et al., 2008). Although genetically obtained ghosts' vaccines are relatively safe, they still have a sort of pathogenicity due to the very little chance of viable cells existence. The full population killing can be achieved by extra processing and manipulation (Haidinger et al., 2003). In this study, we introduce a novel chemical induced BG preparation protocol based on using a critical concentration of a chemical agent for long time.

2. Materials and methods

2.1. Target strain

The bacterial strain targeted to be ghosts was *Salmonella enterica serovar typhimurium* ATCC 13311 obtained from American Type Culture Collection, (VA, USA). The lyophilized cells were reconstituted, then cultured in Muller-Hinton broth (Fluka, Milwaukee, WI, USA) and incubated at 37 °C for 24 h.

2.2. Determination of minimum inhibitory concentration (MIC)

MIC was determined for specific reagents according to the American Society for Microbiology guidelines (Coyle et al., 2005). Stock solutions of Muller-Hinton broth (Fluka, Milwaukee, WI, USA) were prepared containing the following reagents: Tween 80 (5% v/v), SDS (5% w/v), KOH (3% w/v), NaOH (3% w/v), benzoic acid (0.3% w/v) (Lobachemie, Mumbai, India), EDTA (0.2% w/v) (Scharlau, Barcelona, Spain), and lactic acid (0.15% v/v) (WINLAB, East Midlands, England). Tenfold serial dilutions were prepared for each reagent. Aseptically, 100 μl of standard inoculum of S. Typhimurium (matched 0.5 McFarland standard) were added to each diluton. All the tubes were incubated for 24 h before tested for turbidity.

2.3. Preparation of bacterial ghosts

A total number of 9 sterile tubes of 2 mls of Muller-Hinton broth solution supplied with 7% v/v tween 80 were inoculated by 100 μl of standard inoculum of S. Typhimurium. The tubes have divided into 3 groups. Each group involved three tubes which designated for 3 different periods of times: 24, 36 and 48 h. The first group was not treated more. The second group was frozen for one hour at the end of each incubation period. Finally, at the end of each incubation period, the third group was treated by addition of lactic acid (pH = 3.6) and lasting for 20 mins. The third group was examined more by extending the contact time for lactic acid to 30 min, 1, 2 and 3 h. Each experiment was done in triplicate and the results were expressed as average.

Centrifugation was done using (Hettich EBA20S Tuttlingen, Germany) portable centrifuge at 4000×*g* for 10 min. The supernatant was utilized for quantification of proteins and DNA. The obtained pellets were washed by sterile half normal saline solution three times.

2.4. Quantification of released proteins

Bradford method of protein quantification (Bradford, 1976) was used/applied for determination of protein quantities (μg/ml) released by ghost cells using a NanoDrop™ 2000/2000c (Thermo Scientific, MA, USA) spectrophotometer. The standard bovine serum albumin (BSA) provided by the manufacturer was used for generation of standard curve. All readings were taken at 595 nm. The protein contents of the culture media were considered and calculated by measuring the protein contents in the un-inoculated culture media.

2.5. Quantification of released DNA

The NanoDrop™ 2000/2000c -Thermo Scientific spectrophotometer was used for quantification of released DNA in (μg/ml) in the supernatant at 260 nm. Standard concentrations of DNA were used for generation of standard curve. The ratio of absorbance at 260 nm/280 nm was measured to assess DNA purity.

2.6. Scanning electron microscopy (SEM)

The centrifuged pellets of bacteial cells were investegated by SEM (JEOL-JSM-5500 LV): The samples were fixed by glutaraldehyde (2.5%) and dehydrated by serial dilutions of ethanol using automatic tissue processor (Leica EM TP). The samples were dried using CO_2 critical point drier (Tousimis Audosamdri-815). The samples were coated by gold sputter coater (SPI-Module). Finally, samples were examined by SEM with amplification power of x9500 and 20 kV and using high vacum mode at the Regional Center Mycology and Biotechnology, Cairo, Egypt.

2.7. DNA extraction from pellets and supernatant

The DNA was extracted from both pellets and supernatant using AxyPrep™ multisource genomic DNA miniprep kit (Tewksbury, MA, USA). The pellets were separated through high speed centrifugation. The harvested pellets and the supernatant were suspended in TBE buffer, pH = 8. Both pellets and supernatant were processed according to the supplier's specifications.

2.8. Agarose gel electrophoresis

The extracted DNA was analyzed using agarose 0.8% gel electrophoresis separation. Fifteen microliters of DNA extract were mixed with 5 μl DNA gel loading dye (6X) (Thermo Scientific, Waltham, USA). The separated DNA bands were compared with a standard 1 Kb DNA extension ladder marker (Thermo Scientific, MA, USA).

2.9. Gram staining and light microscopy

The centrifuged pellets of bacteial cells were stained by Gram stain then visualized by light microscope using amplification power of 1000 x in order to invistigate cellular external surface integrity.

2.10. Lyophilization of ghost cells

The obtained ghost cells pellets produced from treatments were lyophilized using Christ lyophilizer (Osterode am Harz, Germany)

2.11. Statistical analysis

The significant differnce between the means at a confidence interval of 95% were done using ANOVA and post hoc analysis utilizing IBM based SPSS program version.23.

3. Results

MICs of the following reagents: KOH, NaOH, lactic acid, benzoic acid and EDTA were: 0.0234% w/v, 0.0469% w/v, 0.075%v/v, 0.15% w/v and 0.025%w/v respectively. It was found that the tested serial dilutions of tween 80 and SDS could not inhibit bacterial growth.

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