

Using Mild High-pressure Shock to Generate Bacterial Ghosts of *Escherichia coli*

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Dedicated to Professor Gérard Demazeau on the occasion of his 65th birthday

In the context of vaccine development, bacterial ghosts are inert cells that retain the capacity to activate the immune system, and that can be used as vaccine or carrier for subunit or DNA vaccines. In this study we provide evidence that increasing the copynumber of the *E. coli* K12 *mrr* locus can render naturally occurring and virulent avian pathogenic *E. coli* (APEC) strains hypersensitive to high pressure. We further demonstrate that mild HP shock generates inactive bacterial ghosts from these cells that have not incurred any microscopically visible structural damage. Possible benefits of high-pressure generated bacterial ghosts as a vaccine are discussed.

Key words: Avian Pathogenic *Escherichia coli* (APEC), Hydrostatic Pressure, *Mrr*, Bacterial Ghost

Introduction

Vaccination is a cornerstone of the prophylaxis of infectious diseases and typically makes use of attenuated or inactivated pathogens in the case of whole organism vaccines, or of specific components derived from these organisms, such as surface proteins, in the case of subunit vaccines [1]. Since whole organism vaccines directly serve as antigen for the host's immune system, an essential element during the attenuation or inactivation process is to keep the treated cells as authentic as possible. This allows the immune system to target the antigenic determinants in their native state, *i. e.* the state to which it is normally exposed during natural challenge by the pathogen. However, selection for attenuated variants is often empirical, without understanding the actual molecular mechanism underlying the observed reduction in pathogenicity or virulence. This makes it difficult to predict whether attenuation has a negative effect on the immunogenicity of the vaccine. On the other hand, inactivation of the pathogen can be achieved by heat or chemical treatment, but these treatments may also compromise important antigenic properties.

For this reason, alternative treatments are being investigated that are able to kill bacterial pathogens without any obvious cell damage. In this context, a

promising new technique involves the conditional heterologous expression of bacteriophage PhiX174 lysis gene E, which leads to the formation of a transmembrane tunnel structure through the cell envelope of Gram-negative bacteria. Subsequent leakage of cell constituents generates empty, non-viable cells (or envelopes), termed bacterial ghosts, that nevertheless retain the capacity to trigger the host immune system because they retain an intact cell surface [2, 3]. Moreover, besides their potential as vaccines as such, bacterial ghosts are also interesting delivery vehicles for subunit or DNA based vaccines, which are often poorly immunogenic in pure form, and require strong adjuvants to be sensed by the immune system. This is provided by the tropism of bacterial ghosts, which are taken up very effectively by antigen-presenting cells such as macrophages and dendritic cells. In addition, this also makes them particularly suited as vaccines for mucosal administration by oral, intranasal or aerogenic routes, resulting in the induction of humoral and cellular immune responses [4].

In this paper we examine another mechanism to generate bacterial ghosts, based on high (hydrostatic) pressure (HP) treatment of bacteria. We have recently discovered a molecular mechanism that can be used to sensitise *Escherichia coli* K12 to a mild, sublethal HP shock [5], and here we examine whether this mecha-

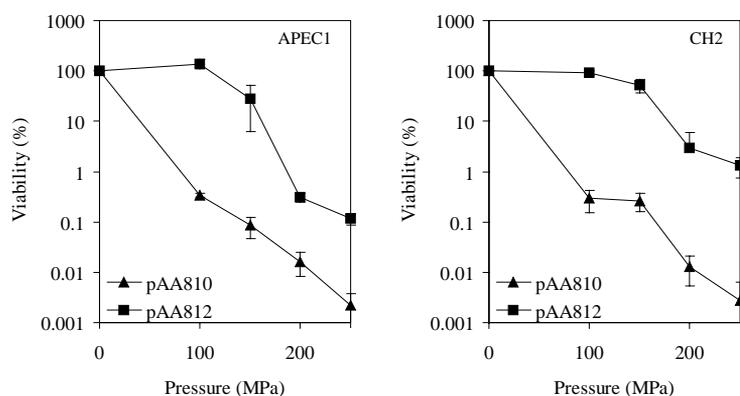


Fig. 1. Inactivation of late exponential phase cultures of APEC strains APEC1 and CH2 after treatment at different pressures (15 min, 20 °C). Strains either harbored plasmid pAA810 (triangles; expressing wild-type Mrr) or pAA812 (squares; expressing an inactive mutant of Mrr). Initial cell concentration was $ca. 5 \times 10^8$ CFU mL^{-1} . Results are expressed as mean \pm standard deviation of three experiments.

nism could be useful to make bacterial ghosts of virulent avian pathogenic *E. coli* strains.

Experimental Section

Strains, plasmids and growth conditions

Virulent avian pathogenic *Escherichia coli* (APEC) strains APEC1 (O45) [6] and CH2 (O78) [7] were used in this study, and transformed with plasmid pAA810 or pAA812 that were constructed earlier [5]. While plasmid pAA810 contains the *E. coli* K12 MG1655 *mrr* gene under the control of its own promoter in the pACYC184 backbone, plasmid pAA812 is isogenic to pAA810 except for a 336 bp in frame deletion in the middle of the *mrr* gene, resulting in expression of an inactive Mrr protein [5]. Transformation of pAA810 and pAA812 to strains APEC1 and CH2 was done by electroporation and subsequent selection on LB agar plates [8] with chloramphenicol (30 $\mu g/mL$).

Overnight cultures of APEC1 and CH2 with the appropriate plasmids were obtained by growth in LB broth for 21 h at 37 °C under well-aerated conditions. Late exponential phase cultures were obtained by diluting overnight cultures 1/100 in fresh pre-warmed LB and allowing further incubation up to an optical density (OD_{600}) of 0.6 as described earlier [9].

High-pressure treatment

For high hydrostatic pressure (HP) treatment, 200 μL of an exponential phase culture was transferred into a sterile polyethylene bag that was heat sealed after exclusion of the air bubbles and subjected to a pressure between 100 and 250 MPa for 15 min at 20 °C. Samples were pressure treated in an 8-mL pressure vessel maintained at 20 °C with an external water circuit (Resato, Roden, The Netherlands). A mixture of glycols (TR15, Van Meeuwen, Weesp, The Netherlands) was used as pressure-transferring liquid. Pressure was built up slowly (approximately 100 MPa/min) to minimize adiabatic heating, while decompression was immediate.

Determination of viability

After HP treatment, the sealed bags were aseptically opened, and pressurized and non-pressurized samples were serially diluted in potassium phosphate buffer (10 mM; pH = 7.0) and surface-plated on LB agar plates. Colonies were counted after 24 h of incubation at 37 °C. Data presented are the mean values from at least three independent experiments, and error bars correspond to standard deviations of the means.

Microscopy

Treated and untreated samples (1.5 μL) were placed on a microscopy glass slide and examined by phase-contrast microscopy at 1000 X magnification with a DMLB microscope equipped with a DC200 digital camera (Leica, Wetzlar, Germany). Images were taken from random fields, and representative photographs are shown.

Results and Discussion

We have recently discovered that the *Escherichia coli* K12 Mrr protein is activated by mild, sublethal HP treatment [5, 10]. Mrr is a cryptic type IV restriction endonuclease that harbors specificity for methylated DNA, although it has no known cognate methylase in the cell [11, 12]. We have found genetic evidence that Mrr generates double strand breaks after mild HP shock, although the exact mechanism behind this HP induced activation of the Mrr protein is still elusive. When in *E. coli* K12 the copy number of its *mrr* locus was artificially increased by providing it on a multicopy plasmid (pAA810), the cells became dramatically sensitive to an HP treatment of 100 MPa (15 min, 20 °C) [5]. The massive inactivation after this otherwise sublethal HP shock presumably stems from an

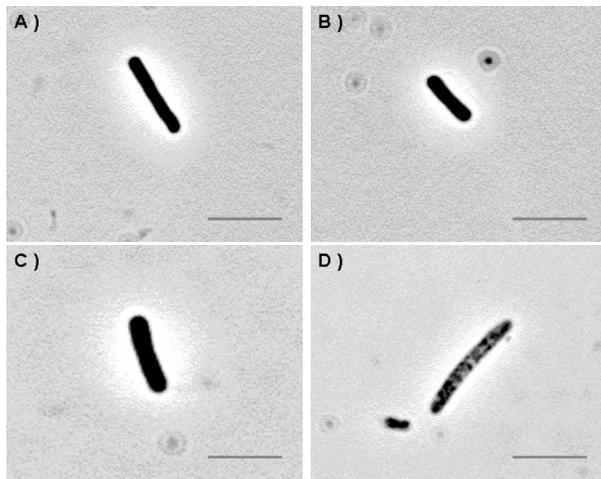


Fig. 2. Photomicrographs of late exponential phase cells of APEC strain APEC1 harboring pAA810 either untreated (A) or pressurized at 100 MPa (B), and of APEC1 harboring pAA812 either untreated (C) or pressurized at 200 MPa (D). Representative photographs are shown. The bar represents 2 μ m. Similar observations were made with APEC strain CH2 (data not shown).

extensive degradation of the bacterial chromosome by the elevated number of activated Mrr proteins.

We wondered whether transforming pAA810 to other strains of *E. coli* could result in a similar HP hypersensitivity. We chose to test our hypothesis in avian pathogenic *E. coli* (APEC) strains, since these pathogens cause colibacillosis in production birds, a common respiratory tract infection that spreads to the internal organs and is responsible for large economical losses in the poultry industry [13]. Moreover, APEC are extraintestinal pathogens (ExPEC) that share many traits with uropathogenic *E. coli* (UPEC), which cause urinary tract infections [14]. APEC strains APEC1 and CH2 were equipped with pAA810 and the pAA812 control plasmid, and subjected to HP ranging from 100 to 250 MPa. Fig. 1 clearly shows that in the presence of functional K12 Mrr both APEC strains become hypersensitive to a 100 MPa treatment, resulting in > 99% inactivation of the population, while cells harboring the control plasmid were unaffected by this pressure. Moreover, to obtain a similar inactivation in the absence of K12 Mrr, cultures had to be subjected to pressures of 200 MPa (APEC1) or higher (CH2) (Fig. 1).

Subsequently we studied general cellular damage provoked by the different treatments by phase contrast microscopy. As a basis for comparison, treatments were chosen that caused at least 99% inactivation as determined by plate count. For the strains harboring pAA810 with the intact *mrr* gene, this was achieved at 100 MPa, while for the control strains with the pAA812 plasmid with the defective *mrr* gene, 200 MPa was required (Fig. 1). Microscopic

analysis revealed that the cells killed by HP alone (200 MPa) were heavily deteriorated, showing a granular cytoplasm, which is an indication of denaturation of cellular proteins and cellular leakage (Fig. 2D). In contrast, cells killed by activation of the Mrr nuclease at 100 MPa were indistinguishable from their untreated counterparts (compare Figs. 2A with 2B). These results indicate that the Mrr-assisted killing induced by treatment at 100 MPa preserves the general cellular structure, which is an essential requirement for generating bacterial ghosts. This is not unexpected, since Mrr activity specifically and only targets DNA.

As can be seen in Fig. 2B, the Mrr-HP generated ghosts retain their refractility, indicating that they are not lysed or permeabilized, unlike ghosts prepared by the earlier mentioned expression of the bacteriophage PhiX174 lysis gene E [2, 3]. The fact to remain sealed is an advantage when the Mrr-HP ghosts are to be used as delivery vehicles for subunit or DNA vaccines, since the recombinant proteins or plasmids do not need to be artificially tethered to the membrane, as is the case with lysed bacterial ghosts [4].

In conclusion, although the mechanism underlying HP activation of the Mrr protein is still obscure, this study provides evidence that the intelligent combination of Mrr and mild HP can be used as a novel strategy to generate bacterial ghosts, which could prove useful in future vaccine development. Moreover, as we learn more about the unique physiological impact of HP on microorganisms, influencing microbial behavior by HP can become an interesting tool in other biotechnological applications.

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