Evaluation of Antibacterial Activity against Salmonella Enteritidis

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Salmonella enterica serovar Enteritidis is a well-known pathogenic bacterium responsible for human gastrointestinal enteritis mainly due to the consumption of eggs and egg-products. The first aim of this work was to study several virulence factors of a strain isolated from egg content: SEovo. First, bacterial growth was studied at several temperatures and cell morphology was observed by scanning electronic microscopy. These experiments showed Salmonella's ability to grow at low temperatures and to produce exoproducts. Next, Salmonella motility was observed performing swimming, twitching, and swarming tests. Results indicated a positive flagellar activity and the cell ability to differentiate and become hyperflagellated under specific conditions. Moreover, SEovo adherence and biofilm formation was carried out. All of these tests enabled us to conclude that SEovo is a potential pathogen, thus it can be used as a model to perform antibacterial experiments. The second part of the study was dedicated to the evaluation of the antibacterial activity of different molecules using several methods. The antibacterial effect of silver and copper aluminosilicates was tested by two different kinds of methods. On the one hand, the effect of these two antibacterial agents was determined using microbiological methods: viable cell count and agar-well diffusion. And on the other hand, the antibacterial activity was evaluated using CLSM and SYTO Red/SYTOX Green dyeing. CLSM allowed for the evaluation of the biofilm on sessile cells, whereas the first methods did not. Results showed that adhered bacteria were more resistant than planktonic counterparts and that CLSM was a good alternative to evaluate antibacterial activity on fixed bacteria without having to carry out a removing step.

Keywords: Salmonella Enteritidis, motility, adhesion, antibacterial tests

Salmonella enterica serovar Enteritidis is a well-known Gram-negative bacillus responsible for foodborne gastroenteritis. Salmonella enterica is a ubiquitous species and has a large range of hosts including fruits, vegetables, pork, poultry, etc. In 2007, in the European Union, 2201 Salmonella outbreaks were identified, 590 of which affected 8922 people. Among the strains involved in these outbreaks, Salmonella Enteritidis was the most frequently serovar isolated (EFSA, 2009). Whether it is on fruits, vegetables, eggs, food-industrial surfaces, or food packaging, Salmonella is attached and can develop microbiological and biofilm structures (Dhir and Dodd, 1995; Humphrey et al., 1995; Joseph et al., 2001). This bacterial state makes Salmonella more resistant against antibacterial agents which could explain its resistance in the previous environments.

For decades, researchers have been working on biofilms and ways to control their proliferation. Kumar and Anand (1998) reviewed several methods to prevent microbial development in the food industry. They list several physical treatments such as ultrasound, high pulsed electrical fields, and UV-light. In more recent years, new methods, such as titanium photocatalysis (Benabbbou et al., 2007) or cold plasma (Niemira and Sites, 2008) have also been studied. Many antibacterial chemical molecules like detergents, chelators, and disinfectants are also well known for their ability to break the biofilm matrix. Among these agents, there are oxidants such as peracetic acid, chlorine, and hydrogen peroxide. Researchers focus their attention on biological molecules such as bacteriocins, enzymes, or antibacterial biopolymers (Kumar and Anand, 1998).

Even if a large range of antibacterial compounds or methods is available, it is not always easy to evaluate precisely their impact on bacterial cells. Several microbiological methods are used to carry out various kinds of antibacterial tests. There are qualitative ones, such as agar-well diffusion and quantitative ones like viable cell count. Other methods are usually practiced. For example: determination of the minimal inhibition concentration (MIC) using microdilution in microplates and BacLight tests (Prakash Singh, 2006). Most of the time, antibacterial activity tests are performed on planktonic cells which are not representative of the in vivo condition. Moreover, bacterial resistance increases in biofilms. Some techniques allow scientists to sample and enumerate biofilms: scraping (Gagnon and Clawson, 1999), swabbing (Joseph et al., 2001), vortexing (Mitchell et al., 2008), or ultrasonic (Doron et al., 2001; Tré-Hardy et al., 2008) before carrying out a viable cell count. However, another kind of technique has emerged in the past decades and enables researchers to study biofilms without further preparation. These techniques are predominantly microscopic, such as confocal laser scanning microscopy (CLSM). CLSM makes the visualization of adhered microorganisms possible. The use of acetic acid labelers enables the study of the impact of an antibacterial agent on biofilms. Indeed, several dyes can be used simultaneously, to evaluate cell viability in the biofilm, according the non superposition of their

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absorption and emission wavelengths, such as SYTO Red and SYTOX Green. The SYTO Red, which is able to diffuse through intact bacterial membranes, labels all bacterial cells (Comas and Vives-Rego, 1997; Biggerstaff et al., 2006). However, SYTOX Green, which is only able to diffuse through damaged membranes, was used to stain dead bacteria (Roth et al., 1997; Lebaron et al., 1998).

The objective of this study was first to demonstrate the pathogenic potential of SEovo. Thus SEovo growth and morphology was observed and bacterial motility determined. The aim of SEovo characterization also consists in studying cell persistence in the environment. CLSM was used to study SEovo adhesion and ability to form biofilm.

The second objective was to use this microorganism of interest to compare several methods to evaluate the antibacterial activity of molecules. On the one hand, microbiological techniques, such as viable cell count and agar-well diffusion methods, were carried out. On the other hand, MCBL was performed. Bacterial viability was determined by live/dead staining on planktonic cells and on adhered cells (Stanimirova et al., 2008).

Materials and Methods

Bacterial strains

The strain used, SEovo, was isolated from eggshell by a French food safety agency (AFFSA) in an egg factory. SEovo was identified as Salmonella enterica serovar Enteritidis. Thus, this strain is typically the kind of bacteria responsible for food-borne diseases due to the consumption of eggs.

Identification of Salmonella

Growth on XLD (Xylose-Lysine-Désoxycholate) agar. SEovo was isolated on XLD agar medium. XLD is a selective medium used to identify Salmonella species. It is low in nutrients and contains a small amount of sodium desoxycholate for selectivity. Salmonella de-carboxylates lysine which keeps the pH neutral or slightly alkaline. At this pH, Salmonella species can produce hydrogen sulphide from the reduction of thioulsphate. This is indicated by ferric ammonium citrate producing black or black-centred colonies.

API 20E strip: API 20E strip was performed on SEovo to confirm that it belongs to Salmonella genus.

Polymerase Chain Reaction: The bacterial culture was pelleted at 13,000 rpm for 1 min, and then heated twice 30 sec by microwaves. The pellet was resuspended in ultra pure water to obtain the DNA sample. The PCR mix was prepared as follows:
- 18.9 µl ultra pure water (Millipore, USA)
- 1 µl DNTPs (25 mM) (Invitrogen, USA)
- 0.25 µl of each oligonucleotide primer (Invitrogen)
- 1 µl magnesium chloride (MgCl2, 500 mM)
- 2.5 µl buffer (Invitrogen)
- 1 µl DNA
- 0.1 µl enzyme (Taq polymerase; 500 U)

The oligonucleotide primer pair used for the universal detection of 16S rRNA gene and mRNA, ICM16SF [5'-CACCGGGGAGGAGGGATGAAAGT-3'] (Eurogentec, Belgium) and ICM16SR [5'-AC CACCGCCCGTCACACCATG-3'] (Eurogentec), was designed by Sheridan et al. (1998) to give a PCR product of 405 bp 16S rRNA. Cycling condition: PCR amplification for 40 cycles (denaturing at 94°C for 45 sec, annealing at 64°C for 45 sec, extension at 72°C for 1 min), with a final extension at 72°C for 10 min. The PCR product was visualized by agarose gel electrophoresis and sequenced by Beckman Coulter Genomics.

Bacterial growth and morphology

Bacterial growth was performed at 37°C, 20°C, and 8°C, corresponding respectively to the optimal Salmonella growth temperature, the environmental temperature, and low refrigeration. Bacterial glycerol stocks were revived in Trypton Soil Broth (TSB) at 37°C under shaking over night in Trypton Soil Broth (TSB) for two successive cycles. The inoculation dose was 6.5×10^7 CFU. Bacterial growth was checked by light absorption at 600 nm. The neperian growth rate was calculated as follows: μ = Ln(2) / G where G is the generation time.

The bacterial morphology was observed at the end of the bacterial exponential phase by scanning electronic microscopy (SEM). The microorganism culture was filtered on a Büchner system equipped with a 0.22 µm filter. Pieces of filter paper were immersed overnight in 5% glutaraldehyde solution and then dehydrated by several washings: phosphate buffer (10 min, 3 times); ethanol 70% (10 min, 3 times); ethanol 90% (10 min, 3 times) and absolute ethanol (10 min, 3 times). Next, the samples were dried by the Carbone dioxide critical point method (Critical Point Dryer BAL-TEC CPD O39) and gold-coated prior to being observed by scanning electronic microscopy.

Motility assays

Swimming: The medium used for the swimming study was Trypton Soy Agar (0.3% agar) containing 2,3,5-triphenyl tetrazolium chloride (TTC) (Fluka, Swiss). TSA was cooled in Petri dishes and 4 µl of an overnight bacterial culture were put on the top of agar media and incubated at 30°C for 24 h.

Swarming: The medium used for the swarming study was LB (20 g/L) with 0.6% Bacto agar and 0.5% glucose. LB agar was cooled in a Petri dish and 4 µl of an overnight bacterial culture were put on the agar media. Petri dishes were incubated at 30°C for 24 h.

Twitching: The medium used for the twitching study was LB agar (1% agar). LB agar was cooled in a Petri dish and 4 µl of an overnight bacterial culture were put on the agar media. Petri dishes were incubated at 30°C for 24 h. The agar media was then removed and the surface of the Petri dish was stained with crystal violet.

Adhesion on glass slide

Adhesion: An overnight culture was pushed off by 7,000 rpm centrifugation and washed with physiological water (0.9% NaCl) three times. The bacterial inoculation quantity was adjusted at 2.10^7 CFU/ml. SEovo was inoculated in a Petri dish containing a glass slide for 2 h at 37°C.

Evaluation of antibacterial activity against Salmonella by microbiological methods

Substances and biocides: Several antibacterial molecules were used to perform antibacterial tests and to check the impact of the method carried out on biocide efficacy. Three aluminosilicates were employed: one containing silver atom (metal percentage unknown), one containing copper (5%) atom and one without metal. These molecules were supplied by an industrial company and their exact composition is confidential.

Agar-well diffusion: Bacterial inoculum (3.25×10^7 CFU/ml) was added to 55°C TSA media (1.5% agar). Medium containing SEovo was cooled in Petri dishes. Wells were then made in the agar media and