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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 biocide 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 microcolonies 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 ultrasounds, high pulsed electrical fields, and UV-light. In more recent years, new methods, such as titanium photocatalysis (Benabbou *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 Slawson, 1999), swabbing (Joseph *et al.*, 2001), vortexing (Mitchell *et al.*, 2008), or ultra sonication (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 acid nucleic labellers enables the study of the impact of an antibacterial agent on biofilms. Indeed, several dyers 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* decarboxylates lysine which keeps the pH neutral or slightly alkaline. At this pH, *Salmonella* species can produce hydrogen sulphide from the reduction of thiosulphate. 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 ($MgCl_2$; 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′-CAGCGGGGAGGA AGGGAGTAAAGT-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: $\mu = Ln(2) / G$ 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 3% 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 twitch plates were inoculated with an overnight bacterial culture with a sterile toothpick at the bottom of the Petri dish. 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 2.10^8 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

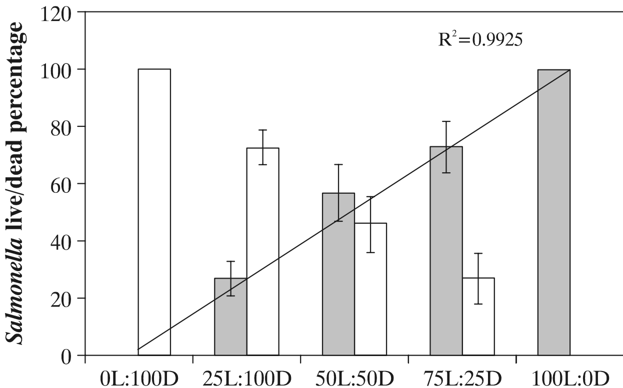

Fig. 1. Calibration of SYTO Red 61/SYTOX Green labeling by CLSM using different live/dead ratio of bacteria. The quantity of dead (□) and live (■) bacteria was determined using Image Tools software: the area covered by red cells (live bacteria) and green cells (dead bacteria) were converted into a percentage of the total area covering.

the antibacterial molecules were put into the wells. Petri dishes were incubated for 24 h at 37°C. After incubation, inhibition of the bacterial growth was evaluated by measuring the diameter of the inhibition ring. The minimal inhibition concentration (CMI) of the silver aluminosilicate was evaluated according to this method. Dilution series were prepared from 5 g/L to 0.05 g/L and then put into the wells.
Viable cell count: An overnight bacterial culture was centrifugated 5 min at 7,000 rpm. Pellet was resuspended in 2 ml of peptone water. The biocide was added to the bacterial solution at 1 g/L and a control experiment was prepared without biocide. Samples were incubated for 1 h at 20°C. Dilution series of the bacterial solutions were prepared, spread on agar media in Petri dishes, and incubated at 37°C for 24 h. Then, colonies were counted.

Evaluation of antibacterial activity against *Salmonella* by Confocal Laser Scanning Microscopy (CLSM)

Planktonic: Biocide was added in excess (10 g/L) to an overnight bacterial culture. The culture with biocide was incubated for 1 h at 20°C. 10 μl of bacterial culture were put between a glass and cover slip, stained, and observed by CLSM.
Biofilm: Bacterial inoculation quantity was adjusted at 6.5×10^7 CFU/ml in TSB in Petri dishes containing glass slides. Petri dishes were incubated for 24 h at 37°C. After incubation, the media was removed and the glass slide was rinsed with physiological water. The biocide solution (10 g/L) was put on the biofilm which had formed on the glass slide. The Petri dish was then incubated for 1 h at 20°C. After incubation, the glass slide was rinsed and bacterial cells were labelled and observed by CLSM.
Observation: Planktonic cells and biofilms were observed with a Leica DM6000B confocal microscope (Leica Microsystems, Germany) with the immersion X40 objective. For the evaluation of antibacterial activity against *Salmonella*, cells were stained with SYTO Red 61 (10 μM) (Molecular Probes) which labelled all the bacterial population and SYTOX Green (0.25 μM) (Molecular Probes) which labelled only the damaged cells. For SYTO 61, the excitation wavelength was 633 nm (Helium/Neon laser) and the emission was collected from 650 nm to 750 nm. For SYTOX Green, the excitation wavelength was 488 nm (Argon laser) and emission was collected from 500 nm to 550 nm. Calibration of the taggers was carried out with several bacterial live/dead ratio stained by SYTO Red and SYTOX Green and observed by CLSM. A regression line was obtained with a regression coefficient of 0.9925 (Fig. 1).

Images were analyzed with Image Tools software as grey-scale interpretations on the screen. The area covered by the cells was converted into a percentage of the total area covering.

Results and Discussion

Salmonella Enteritidis characterization

The first step of the characterization was to identify SEovo. SEovo growth on XLD medium showed the development of yellow colonies with black centres. This observation forwarded the hypothesis that this strain was actually *Salmonella* spp. A second method, API 20E strip, was used to prove it. Based on biochemical reactions, API 20E strip made the identification *Salmonella* spp. possible. This experiment confirmed that SEovo was *Salmonella* spp. with an identification which was very good (84.8%). Finally, a PCR was carried out on 16S RNA. The sequencing revealed that SEovo was *Salmonella enterica* subsp. *enterica* serovar Enteritidis with 87% identification.

Next, SEovo growth was studied at 37°C, 20°C, and 8°C. The neperian growth rates were respectively 0.8, 0.45, and 0.23 (per h). Results showed that bacterial growth was affected by low temperature but not inhibited. Indeed, at 8°C, SEovo was still able to grow and have a metabolic activity. The latency period increased at 8°C and 5 h were necessary before the exponential period. Matches and Liston (1968) also observed the increase (12 days) of the latency period of *Salmonella* with a temperature decrease from 8.3°C to 6.7°C. This observation suggested a cell adaptation against low temperature: new met-

Table 1. Characterization of SEovo growth, morphology, motility behaviour, and adhesion ability

		SEovo
Growth		
Neperian growth rate	at 37°C (/h)	0.8
	at 20°C (/h)	0.45
	at 8°C (/h)	0.23
Morphology	SEM	
	Size (μm)	1.56±0.25
	Exoproducts	+
Motility	Swimming	+
	Twitching	-
	Swarming	+
Adhesion on glass slide	Covering percentage	6.6±1.2

SEM showed cell size and presence (+) or absence (-) of exoproducts. Swimming, twitching and swarming test showed the motility ability of *Salmonella*: (+) ability (-) inability. Cell adherence was determined using CLSM and SYTO Red 61 dyeing. The area covered by stained cells was converted into a percentage of the total area covered using Image Tools Software.

abolic routes and more adapted enzymes (Matches and Liston, 1968). This first experiment demonstrated the ability of SEovo to adapt itself, to survive, and grow under low temperature environment.

SEM studies made the description of the bacterial morphology possible; cell size was measured and indexed in Table 1. The average size of SEovo was 1.5 μm long. SEM pictures of SEovo showed the presence of exoproducts which might be thin aggregative fimbriae (Tafi). Tafi, which is also called curli, are associated with the bacterial adherence and biofilm formation (Castonguay *et al.*, 2006; White *et al.*, 2006). White *et al.* (2006) have also shown that fimbriae confer to *Salmonella* Enteritidis stress resistance against desiccation and antibacterial agents like hypochlorite.

Moreover, this observation led us to hypothesize that the *Salmonella* strain studied had other appendixes responsible for bacterial motility, adhesion, and biofilm formation, such as flagella and pili.

Thus, several motility tests, swimming, twitching, and swarming, were performed to identify the activity of flagella and type IV pili. Indeed, bacterial swimming ability revealed the positive activity of flagella and twitching ability was due to the extension and retraction of type IV pili causing cell propulsion on a surface. Swarming is a particular bacterial behaviour expressed in specific environmental conditions resulting from bacterial differentiation: cell extension, hyperflagellation, and the production of extracellular compounds, such as biosurfactants and polysaccharide. Results showed that SEovo was able to swim, but not to twitch, which means that this strain had a negative type IV pili activity and a positive flagellar activity. Type IV pili and flagella, are not only responsible for bacterial motility, but are also involved in the first stages of bacterial adhesion and biofilm formation (O'Toole and Kolter, 1998; Robertson *et al.*, 2003). Robertson *et al.* (2003) studied *Salmonella* Enteritidis adhesion in the gastrointestinal tract of the rat according to flagella expression. Their experiments showed that *Salmonella* Enteritidis, which did not express flagella, adhered less than those which did express flagella and could not persist in the gastrointestinal tract. Moreover, flagella are important invasiveness factors responsible for host inflammatory response. Indeed, flagella are likewise type III secretion systems. This needle system delivers proteins into the host cells and generates an inflammatory response (Kubori *et al.*, 2000; Josenhans and Suerbaum, 2002). Moreover, results revealed that in specific environmental conditions, SEovo was able to swarm. Swarming behaviour results from quorum-sensing communication leading to multicellular behaviour and bacterial differentiation. Thus, this behaviour is closely linked to biofilm formation and bacterial virulence (Sharma and Anand, 2002), which suggestes that SEovo had an important pathogenic potential.

To complete this part of the work, the adherence of *Salmonella* Enteritidis was determined by measuring the percentage of the glass slide which was covered by the bacteria. Indeed, bacterial adhesion is the first step of biofilm formation. The results showed that SEovo was able to adhere on glass slides and covered 6.6% of the surface. This ability could be explained by the production of curli. Cookson *et al.* (2002) studied the adherence and the biofilm formation of a wild type *Escherichia coli* which overproduced curli fimbriae and a mutant which did not produce curli. They showed that bacteria which produced curli had a better adherence on abiotic surfaces and better biofilm formation than the strains which did not produce curli. This was correlated with the observation made for SEovo. Indeed, SEM showed the production of exoproducts which might be curli. Moreover, flagellar activity is also in-

Table 2. Influence of aluminosilicate compounds on SEovo

	Silver aluminosilicate	Copper aluminosilicate	Empty aluminosilicate
Planktonic condition			
Diameter inhibition zone (mm)	18.7±0.21	0	0
Viable cell count (log reduction)	1	-	-
MIC (g/L)	0.08	-	-
CSLM	75 μm	75 μm	75 μm
dead bacteria percent	70%	24%	22%
Adhered condition			
CSLM	75 μm	75 μm	75 μm
dead bacteria percent	9%	12%	2%

The impact of silver and copper aluminosilicate on SEovo was observed in the planktonic condition using the agar-well diffusion method and confocal microscopy, as well as on adhered cells. CLSM photography shows the ratio of live and dead cells. Dead bacteria percentages were determined using Image Tools Software. (-) Test not performed.

volved in bacterial adhesion. Thus, this observation was in accordance with the previous test on motility.

The phenotypical characterization of SEovo highlighted the expression of virulence factors and behaviour involved in the contamination of surfaces. Thus, this strain was a good model to compare different methods to evaluated antibacterial agents.

Methods to evaluate antibacterial activity against *Salmonella* Enteritidis

The aim of the second point of the study was to compare several methods to evaluate the antibacterial activity of molecules and the impact of the physiological state of the bacteria on the efficacy of antibacterial compounds.

Firstly, classical methods were performed on SEovo, such as agar-well diffusion methods and viable cell counts, to determine the impact of several aluminosilicates. Next, the antibacterial activity was evaluated by CLSM. The agar-well diffusion method enabled the demonstration of the influence of the antibacterial agent on the growth of SEovo. After 24 h incubation at 37°C of the agar medium containing SEovo, an inhibition zone was observed around the well containing silver aluminosilicate; the diameter measured of the well was 1.87± 0.21 cm (Table 2). However, any growth inhibition was observed for copper aluminosilicate and aluminosilicate without metal. Agar diffusion methods also permitted the evaluation of the MIC. The determination of MIC was carried out for the silver aluminosilicate only and was 0.08 g/L. To quantify the impact of the silver aluminosilicate and on bacterial viability, the viable cell count method was performed. The silver molecule caused 1 log (CFU/ml) decrease. In this study, the silver aluminosilicate had a better antibacterial activity than copper aluminosilicate and the aluminosilicate without metal. Both of these microbiological methods, agar-well diffusion and viable cell count, commonly used to evaluate the antibacterial activity of a molecule, are complementary and give information about the impact of a molecule on bacterial growth and bacterial viability. However, these methods cannot be performed in the case of adhered cells.

Secondly, antibacterial activity of the several was observed on planktonic and sessile cells by CLSM. *Salmonella* was in contact with the agent for 1 h at 20°C before being stained with SYTO/SYTOX and observed by CLSM. Results are reported in Table 2. Silver, copper, and empty aluminosilicate induced respectively 70%, 24%, and 22% bacterial death. These results correlated with the agar-well diffusion method. Next, experiment was performed on adhered cells. SEovo was adhered on glass slides for 24 h. Antibacterial agent was then put on *Salmonella* adhered cells for 2 h. In this physiological state, silver aluminosilicate caused only 9% of bacterial death, while copper aluminosilicate and empty aluminosilicate caused, respectively, 13% and 2% of the bacterial death (Table 2). These results give clear evidence that adhered *Salmonella* Enteritidis is more resistant against antibacterial agents than in the planktonic state. Several research teams (Doron *et al.*, 2001; Tré-Hardy *et al.*, 2008; Kubota *et al.*, 2009) have previously shown the same difference of resistance depending on the physiological state of the bacterial cells: these studies were carried out on *Streptococcus sobrinus*, *Pseudomonas aeruginosa*, and *Lactobacillus plantarum* subsp. Moreover, in a large range of cases, bacteria are adhered on a surface. It is therefore important to evaluate potential antibacterial agents on adhered cells. This study showed that confocal microscopy is a method which enables these kinds of tests using live/dead staining.

To conclude, this work gave evidence that the strain studied, SEovo, expressed several virulence factors. Indeed, the first step of this study showed bacterial resistance and growth at low temperatures, expression of curli and flagella, and ability of the strain to adhere on surfaces. Thus, this strain was used as a potential pathogen to study the antibacterial activity of several molecules using microbiological methods and confocal microscopy. Results showed that silver aluminosilicate was the most efficient antibacterial agent. However the bactericide effect was only shown on planktonic cells. Indeed, this study demonstrated that *Salmonella* Enteritidis was more resistant in a sessile state than in a planktonic state. Indeed, several studies have demonstrated that bacteria in adhered conditions are more resistant to antibacterial agents than in a planktonic state (Joseph *et al.*, 2001; Teitzel and Parsek, 2003). This is an important observation that needs to be considered to evaluate properly antibacterial activity. The evaluation of an antibacterial agent only on planktonic cells is not sufficient. The viable cell count method can be performed on adhered bacteria, but it requires a cell removing step. Several techniques exist to remove adhered bacteria: the most common methods are scraping with a knife (Gagnon and Slawson, 1999), swabbing with cotton (Joseph *et al.*, 2001), sonicating (Doron *et al.*, 2001; Tré-Hardy *et al.*, 2008), and vortexing (Mitchell *et al.*, 2008). Each of these methods has some inconvenience: scraping and swabbing are subject to individual variation; sonication and vortex methods can damage the cell integrity and impact the results. In this study, we suggest using CLSM to determine the antibacterial potential of a compound, both on sessile cells and the planktonic counterpart.

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