Viable but Nonculturable *Salmonella typhimurium* in Single- and Binary-Species Biofilms in Response to Chlorine Treatment

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**ABSTRACT**

The survival after chlorine stress of the biofilm form of *Salmonella typhimurium* both alone and in association with the biofilm form of *Pseudomonas fluorescens* was investigated. *P. fluorescens* showed the best adhesion and more extended growth than *S. typhimurium* when the two strains were cocultured. The presence of *P. fluorescens* resulted in an increased resistance of *S. typhimurium* to chlorine. This phenomenon, which was already seen in 1-day biofilms, increased in 4-day biofilms. Viable but nonculturable cells were observed only in 4-day single-species *S. typhimurium* biofilms subjected to chlorine stress; only 50% of substrate-responsive bacteria (SRB) were culturable. When daily cycles of disinfection, neutralization, and culture medium supply were performed with *S. typhimurium* biofilms for 4 days, only 20% of the SRB remained culturable. The chlorine consumption of such biofilms was more than twice that of 4-day single-species *S. typhimurium* biofilms.

Key words: Biofilm, disinfection, chlorine, nonculturable bacteria, *Salmonella typhimurium*

The effectiveness of disinfection procedures was first determined using planktonic microorganisms, and this determination is still used in standardized methods (1). However, it has now been widely demonstrated that microorganisms are far more resistant when in a biofilm (7). Moreover, the best disinfectants for planktonic cells are not necessarily the best for biofilm cells (13).

Furthermore, studies on biofilm disinfection usually do not take into account two major facts. The first is that disinfection can result in the appearance of viable but nonculturable bacteria (11, 29), whose culturability can be recovered when they are placed in a favorable environment (28). However, disinfection efficiency is usually assessed by determining the numbers of culturable bacteria. The second fact is that the microflora of food-industry surfaces is a complex community, contrary to the laboratory pure-species biofilms usually used to study disinfectant efficiency. Psychrotrophic pseudomonads are largely dominant on food-preparation surfaces in the catering industry (3), where cold temperatures are maintained. These bacteria have a high capacity to colonize surfaces, which is probably associated with their ability to produce exopolymeric substances (EPS). In addition, they can play a role in helping other species to colonize surfaces. Indeed, Sasahara and Zottola (26) could not obtain an extended biofilm with *Listeria monocytogenes* if it was not associated with *Pseudomonas fragi*.

These two facts led us to study the sensitivity to chlorine of *Salmonella typhimurium* cultivated in single-species biofilms or in association with *Pseudomonas fluorescens*. Bacterial survival was assessed by plate count and by the direct viable count (DVC) method of Kogure et al. (19), in which viable bacteria whose growth is inhibited by nalidixic acid are able to elongate in the presence of yeast extract. These viable bacteria are also called substrate-responsive bacteria (SRB) (23).

**MATERIALS AND METHODS**

*Bacteria*

*P. fluorescens* CIP 56-90 was obtained from the Collection de l’Institut Pasteur (Paris, France) and a rifampicin-resistant strain of *S. typhimurium* from the CNEVA, Ploufragan (Laboratoire Central de Recherches Avicoles et Porcine, Ploufragan, France). Long-shelf- life stock cultures were stored in 2-ml tubes at −20°C in a glycerol medium according to the French Standard NF T72-140 (2). Every month, a stock culture was prepared for each strain: frozen bacteria were placed at their optimum growth temperature (25 and 37°C for *P. fluorescens* and *S. typhimurium* respectively); after 16 h of incubation they were transferred to tryptic soy agar (TSA, Difco) slopes and incubated at their respective optimum growth temperatures for 20 h before being placed at 3°C. The day before an experiment, refrigerated cultures were transferred to TSA slopes and incubated for 20 h at their optimum growth temperature.

**Biofilm substratum**

New polycarbonate slides (50 by 25 by 1 mm) (Goodfellow, Lille, France) were washed just before use according to the procedure described by Bellon-Fontaine and Cerf (4), slightly modified. The slides were washed by a 10-min immersion under agitation in 500 ml of an aqueous 2% (vol/vol) RBS35 (Société de traitement chimique des surfaces, Frelinghien, France) solution. The initial temperature of this solution was 50°C. The slides were then rinsed by immersion...
in 500 ml of tap water (initial temperature 50°C) with agitation for 25 min. Five more 1-min immersions with agitation in 500 ml of distilled water at ambient temperature were performed. The slides were then autoclaved (121°C, 20 min) and dried in a laminar air flow hood.

**Biofilm development**

Biofilms were developed using a modification of the procedure of Maris (22). Bacteria were washed twice by centrifugation (2800 x g, 10 min) in 9 ml of physiological saline. Suspensions in physiological saline containing approximately 2 x 10^6 CFU ml^-1 (OD620 = 0.29 in 1.5-cm diam tubes) were made. Inocula were prepared as follows: to obtain a pure-culture biofilm, 2 ml of the S. typhimurium suspension were diluted with 2 ml of physiological saline; to obtain a mixed-culture biofilm, 2 ml of each of the two bacterial suspensions were mixed together. A 50-µl inoculum was deposited on a clean polycarbonate slide which was then placed in a humidity cabinet (LHL-212ME, Espec, Bioblock, Illkirch, France) at 25°C and 95% relative humidity (RH) for 3 h to allow adhesion to occur. The nonadherent bacteria were then removed by pouring 25 ml of saline peptone (Tryptone-sel, AES Laboratoire, Combourg, France) over the place where the bacteria had been deposited. As culture medium, 200 µl of saline peptone was deposited on the adhering bacteria before incubation at 25°C and 95% RH for 20 h. A final rinse with 25 ml of saline peptone was used to eliminate nonbiofilm bacteria; this preparation constituted a 1-day biofilm. Four-day biofilms were obtained with the same incubation conditions by adding new culture medium every day after rinsing as above on three more days.

With this protocol a circular area of 1.1 cm² was colonized for all the biofilms studied except for the 4-day binary-species biofilm, which colonized an area of 1.3 cm².

**Biofilm disinfection**

The biofilm was immersed for exactly 5 min in 30 ml of a sodium dichloroisocyanurate (Javel solide, Mercier, Villeneuve d'Asq, France) solution containing 5.5 mg l⁻¹ of free chlorine (pH 6.6) at 23°C. Those conditions had been determined previously in order not to inactivate all bacteria. After the 5-min contact time the biofilm was immediately transferred to 30 ml of a sterile neutralizing solution containing 17 g l⁻¹ of sodium thiosulphate (Labosi, Maurepas, France) and was maintained immersed for 5 min.

In order to better simulate what happens in the food industry, daily disinfection was also performed on biofilms for four days. The culture medium was then deposited on the biofilm just after neutralization to allow for regrowth of the residual microorganisms.

**Free chlorine assay**

The diethyl-p-phenylenediamine method described by Rodier (25) was used. This method consists in adding diethyl-p-phenylenediamine (Sigma) to the solution to be analyzed. Free chlorine concentration is calculated from the volume of ferrous ammonium sulphate (Labosi, Maurepas, France) solution needed to make the red color disappear.

**Sonication treatment to detach bacteria from biofilm**

The following procedure has been adopted after a previous study (results not presented) on optimization of the recovery of biofilm bacterial cells. Biofilm immersed in a polyethylene flask containing 35 ml of saline peptone was placed for 4 min in a 28 kHz, 2 x 150 W sonication bath (Delta 220, Deltasonic, Meaux, France) with the water maintained at 40°C. The slides were then rinsed by pouring 50 ml of saline peptone over the place where the biofilm was to eliminate the residual nonadhering bacteria, followed by TSA to verify whether all the bacteria were removed. It was observed that the colony-forming units (CFU) in the last rinse never exceeded 0.2% of the biofilm population, and residual contamination of the slides never exceeded 30 CFU. Hence, and as no aggregate could be found by microscopic examination of the suspension obtained after sonication, it can be concluded that biofilm populations obtained by this method were not underestimated.

**Determination of colony-forming units**

Colony-forming units (CFU) were determined with a spiral plater (Spiral System® DS, Interscience, France). P. fluorescens plate counts were performed on TSA supplemented with 0.4% (vol/vol) CFC (cetrimide-fucidine-cefaloridine) (Oxoid) and S. typhimurium counts on TSA supplemented with 0.67% (vol/vol) of a 13.5 mg l⁻¹ rifampicin (Sigma) methanol solution. Colony counts were determined after a 24-h incubation at 25°C for P. fluorescens and at 37°C for S. typhimurium.

**Acridine orange direct counts**

Bacterial suspensions obtained after biofilm sonication were diluted, if necessary, in an appropriate volume of distilled water to obtain a final concentration of at least 15 cells per microscopic field, according to the recommendations of Trousselier et al. (30). Acridine orange (Rhône Poulenc, Villers St. Paul, France) was added to the diluted suspension to a final concentration of 0.01% (wt/vol) for a contact time of 5 min. An appropriate volume was then filtered through a 0.22 mm pore size black polycarbonate membrane (Nuclepore® 13-µm diameter, Schumacher DMF, Gonesse, France) which was then rinsed with 5 ml of filtered distilled water and mounted on a glass microscope slide. Slides were examined with a microscope (standard model; Carl Zeiss) equipped with an epifluorescence system. A minimum of 20 fields were observed.

**Determination of direct viable count of S. typhimurium**

The direct viable count (DVC) described by Kogure et al. (19) was combined with the indirect fluorescent-antibody (IFA) procedure. Conditions of the DVC method were previously determined to obtain maximal elongation of S. typhimurium: nalidixic acid (crystalline sodium salt, Sigma) and yeast extract (bioMérieux, Marcy l’etoile, France) were added to the bacterial suspensions obtained after biofilm sonication to a final concentration of 0.001% (wt/vol) and 0.025% (wt/vol) respectively. The mixture was incubated for 24 h at 37°C. An appropriate volume of this suspension was then filtered through a 0.22 µm pore size black polycarbonate Nuclepore® membrane. The IFA procedure was performed as described by Desmonts et al. (11): a 20-µl sample of Salmonella test serum Polyvalent 1 serogroups A-E4 obtained from immunized rabbit (Behring, Rueil-Malmaison, France) was deposited on the membrane, which was incubated for 30 min at 37°C; the membrane was then rinsed with 5 ml of phosphate-buffered saline; 20-µl of fluoroisothiocyanate conjugate (anti-rabbit IgG FITC conjugate, F(ab')2, Sigma) were deposited on the spot where the drop of serum had previously been placed; the membrane was then incubated and rinsed as previously. The membrane was mounted on a glass microscope slide. A minimum of 300 elongated cells were counted under the epifluorescence microscope.

**Data interpretation**

Disinfection efficiency (E) is defined as the number of decimal reductions of the CFU or SRB counts: E = log10[N0/Nf], where N0 is the initial count of bacteria and Nf the final number of bacteria after disinfection.

**Statistical analysis**

The statistical analysis was performed with Statgraphics software (version 6, Manugistics™, Maryland, USA).
RESULTS

Effect of P. fluorescens on the adhesion of S. typhimurium

Cells from the bacterial suspension which were deposited on the surface during 3 h of incubation and were not removed by rinsing were considered to be adherent. The numbers of adherent CFU were determined after detachment of the cells by sonication. As it has been verified that no growth occurred in physiological saline during the 3-h period, one can consider the percentage of adherent cells as a good indicator of bacterial adhesion. No significant difference between the adhesion of the two single-species suspensions could be seen (Fig. 1). On the other hand, when the suspension contained the two bacterial species, analysis of variance showed that P. fluorescens significantly hindered (P = 0.03) adhesion of S. typhimurium. No significant difference could be established for P. fluorescens adhesion between single- and binary-species suspensions.

![Graph showing adhesion of P. fluorescens and S. typhimurium](image)

Figure 1. Adhesion to polycarbonate of P. fluorescens (P) and S. typhimurium (S) present in single- and binary-species suspensions. Error bars represent standard deviations (2 duplicate trials).

Effect of P. fluorescens on the growth of S. typhimurium

Two different categories of cells were enumerated: those that resisted the rinse and were considered biofilm cells and those that were removed by the rinse and were called planktonic cells. The two single-species cultures had equivalent biofilm and planktonic cell populations (Table 1). In mixed culture the growth of S. typhimurium was reduced for both planktonic and biofilm populations by a factor of 10, whereas the growth of P. fluorescens remained unchanged.

![Graph showing growth of bacteria](image)

Comparison of DVC and CFU counts of S. typhimurium in intact biofilms

With intact biofilms the two enumeration methods gave the same results whatever the type (single or binary species) and age of biofilm (Table 2). The total acridine orange direct count for the 1-day S. typhimurium biofilms was also determined in 2 duplicate trials (7.52 ± 0.09) and showed no difference from the 2 other determinations. These results proved that all the biofilm cells were culturable and substrate-responsive and that sonication allowed for good separation of biofilm bacterial cells without modifying their culturability.

![Graph showing disinfection efficiency](image)

Figure 2. Disinfection efficiency (E) of chlorine on S. typhimurium within single- or binary-species 1- and 4-day biofilms. Error bars represent standard deviations: 3 CFU and 2 SRB duplicate trials for 1-day biofilms; 2 duplicate and 1 single trial for 4-day biofilms. SRB, substrate-responsive bacteria.

Disinfection of S. typhimurium cultivated in single-species biofilms or associated with P. fluorescens

For 1-day biofilms there was no visible difference between disinfection efficiency (E) assessed with CFU or SRB (Fig. 2). Analysis of variance showed that disinfection efficiency against S. typhimurium appeared lower (P = 0.03) when the bacteria were cultivated in binary species biofilms, where E = 2.8, than when cultivated in single species biofilms, where E = 3.3. That means a 15% decrease of disinfection efficiency.
On 4-day biofilms E was always less than 1 (Fig. 2), confirming the well-known observation that biofilm resistance increases with biofilm age (13, 20). Again, the presence of \textit{P. fluorescens} increased \textit{S. typhimurium} resistance \((P = 0.006)\), and the phenomenon was slightly greater than in the 1-day biofilms, as the decrease in \(E\) between single- and binary- species biofilms was here 57\%. Analysis of variance also showed a significant difference between efficiency measured by CFU reduction and by SRB reduction, but this did not exist only when \textit{S. typhimurium} was cultivated in single-species biofilms (paired-means comparison, \(P = 0.04\)).

**Comparison of loss of culturability between 4-day biofilms disinfected-daily and disinfected once**

Means comparisons showed significant differences in culturability (expressed as the percentages of cultivable cells [CFU] among the SRB) between intact 4-day single-species \textit{S. typhimurium} biofilms and those disinfected once \((P = 0.035)\) and between intact 4-day single-species \textit{S. typhimurium} biofilms and those disinfected daily (\(P = 0.004\)) (Fig. 3). The loss of culturability was greater when the biofilms had been disinfected daily (\(P = 0.05\)). On the other hand, no loss of culturability could be observed in binary-species biofilms.

**Comparison of chlorine consumption between biofilms disinfected daily and biofilms disinfected on the 4th day**

Chlorine consumption increased every day when both single and binary species biofilms were disinfected daily for 4 days and was finally much greater than the chlorine consumption of the 4-day biofilms (Table 3).

**DISCUSSION**

When mixed-species suspensions were used, \textit{P. fluorescens} was dominant after adhesion. Incubation temperature could at least partially explain this phenomenon. Of course the physicochemical characteristics of cells surfaces are implicated in adhesion, and exopolysaccharide conformation can be modified by temperature shifts (10); but, also it seems that adhesion may require metabolic activities, i.e., protein synthesis (12). Bacterial adhesion is influenced by temperature (14, 18). Shea et al. (27) showed that adhesion of the marine bacterium \textit{Deleya marina} to a hydrophilic substratum peaked at 25°C, the optimum temperature for growth. Here, the incubation temperature of 25°C is the optimum temperature for growth of \textit{P. fluorescens}, whereas the optimum growth temperature of \textit{S. typhimurium} is 37°C. After growth, \textit{P. fluorescens} still dominated both in the biofilm and in the planktonic cells, surely because its growth rate is greater than that of \textit{S. typhimurium} at 25°C. In this case the poor culture medium was preferentially used by \textit{P. fluorescens} and was exhausted before \textit{S. typhimurium} could reach the same population as in single-species culture.

The result of the assumed competition for nutrients in mixed biofilms would be that \textit{S. typhimurium} did not have the same nutritional history in single-species as in mixed-species biofilms. It is well known that nutrient deficiency increases resistance to antimicrobial agents (3, 16). This could explain why \textit{S. typhimurium} was more resistant when associated in biofilms with \textit{P. fluorescens}, this phenomenon being enhanced in 4-day biofilms by a longer state of nutrient deficiency.

Another fact that could explain the increased difference in resistance between single- and binary-species biofilms when biofilms get older is an increase in cell density. Indeed, it has been reported that an increase in cell density enhances the resistance of a \textit{P. aeruginosa} biofilm to iodine (6). Here, cell density (expressed as CFU cm\(^{-2}\)) of single- and binary-species biofilms was not different for the 1-day biofilms (Table 1), but in the 4-day binary-species biofilms it was three times greater than in its single-species counterpart (result not presented).

Bacterial cells are known to produce very little chlorine demand (27). Bacterial chlorine demand is essentially linked to extracellular polysaccharides (9) or extracellular capsular material (20). The chlorine consumptions of single- and binary-species biofilms were different. Therefore, it seems unlikely that EPS were responsible for the different resistance of \textit{S. typhimurium} to chlorine, unless different biochemical properties of the EPS of the two species were involved.

One could not detect any loss of culturability in intact biofilms even in the 4-day biofilms. Those biofilms grown in

**Comparison of chlorine consumption between 4-day biofilms disinfected once and 4-day biofilms disinfected daily**

Comparison of chlorine consumption between 4-day biofilms, which were disinfected daily and biofilms disinfected on the 4th day, showed no significant difference in chlorine consumption (Table 3).

**Table 3. Chlorine consumptions during the 5-min contact time of daily and fourth-day disinfected single- and binary-species \textit{S. typhimurium} and binary-species 4-day biofilms**

<table>
<thead>
<tr>
<th>Biofilm age (days)</th>
<th>Biofilms disinfected daily</th>
<th>Biofilms 4-day disinfected</th>
<th>Single species</th>
<th>Binary species</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>1.09</td>
<td>1.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.50</td>
<td>2.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2.70</td>
<td>2.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3.40</td>
<td>2.63</td>
<td>1.36</td>
<td>1.20</td>
</tr>
</tbody>
</table>

\(a\) Mean values from duplicate tests; standard deviation was always less than 0.1.
poor culture medium were thin and nutrient diffusion was probably not hindered as in thick biofilms (8) where viable but nonculturable cells could be found because of starvation (17).

Losses of culturability were observed only after chlorine treatment in the 4-day S. typhimurium biofilms: nearly 50% of the viable cells were culturable when the biofilms had been disinfected once, whereas only 20% of the viable cells were culturable when the biofilms had been disinfected daily. The loss of culturability was observed only in aged biofilms where chlorine was much less aggressive, most probably because of the increase in the EPS content of the biofilm. This leads one to conclude that chemical stress need not be too drastic to allow for the appearance of nonculturable bacteria. As further evidence, when the stress was repeated daily, an increase in biofilm chlorine consumption (i.e., an increase in protective EPS content) was concomitant with a further decrease in culturability. The increase in EPS content of biofilms disinfected daily can be looked at as an adaptation to chlorine because of the protective effect of EPS. Such an increase in exopolysaccharides was observed by Robertson and Firestone (24) as an adaptation to desiccation. The authors showed that the purified exopolysaccharides could retain water amounting to several times their mass. The polymer matrix could be regarded here as a water-laden gel which protects the microbial cells from desiccation.

Loss of culturability was not observed in binary-species biofilms, perhaps because the small chemical stress (E < 0.5) was not sufficient to allow mechanisms leading to the appearance of viable but non culturable bacteria.

In conclusion, this work shows that environmental conditions, the history of the biofilm bacteria, and the enumeration method are of great importance in assessing disinfection efficiency. All the stresses to which surface bacteria in the food industry are subjected (chemical stress, desiccation, starvation, etc.) seem to be conducive to increased bacterial resistance to disinfection in comparison with laboratory-cultivated biofilm bacteria. Nonpsychrotrophic pathogens (most of the foodborne pathogens), if not numerous because of low temperatures maintained in food industry premises, are probably all the more resistant to disinfectants because they have been subjected to adverse temperatures. A so-called stressed state, or an injured one, is most probably the natural physiological state of food-surface bacteria. Food hygienists, as microbiologists studying surface or drinking water already do, should take this fact into account.

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