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On-Line Visualization of the Competitive Behavior of Antagonistic Bacteria

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To study the interaction between cocultured *Listeria monocytogenes* and an antagonistic *Leuconostoc* strain producing an anti-*Listeria* bacteriocin, flow cytometry, a technique allowing on-line and real-time analysis, was used along with classical microbiological methods. Culture methods and flow cytometric measurements of the mixed culture over time point to a bactericidal action of the lactic acid-producing bacterial strain against *L. monocytogenes* cells.

Many bacteria produce protein compounds, named bacteriocins (7, 13), that are detrimental to other bacterial species present in their environment. The colicins synthesized by various *Escherichia coli* strains have been widely studied, and in many cases, their mode of action is known (8, 10). However, in spite of increasing efforts, much less is currently understood about the activity of the bacteriocins produced by lactic acid-producing bacteria (7). A lactic acid-producing bacterial species, *Leuconostoc mesenteroides* Y105, was found to inhibit the growth of *Listeria monocytogenes* in a well diffusion method generally used to isolate such strains (6, 14). Growth inhibition is mainly due to the secretion into the culture medium of a protein compound, mesentericin Y105, specifically directed toward *L. monocytogenes*, i.e., not affecting any other lactic acid-producing bacteria tested to date (5). However, although the well diffusion approach permits the screening of antagonistic strains from selected natural sources, such as dairy products, it does not provide sufficient information about how they interact. Whether the lactic acid-producing cell has a bactericidal or bacteriostatic effect against the target cell cannot be assessed rapidly. Moreover, this method is limited to solid medium (agar plates); liquid mixed cultures cannot be analyzed directly.

Because flow cytometry is an efficient method, used mainly to study or sort eukaryotic cells, we focused on this technique to perform our study, even though, to our knowledge, it has never been used this way before. We believed a priori that different types of cells could be detected if they displayed different morphological characteristics (1). This is obviously the case when comparing *Listeria* (short rods) and *Leuconostoc* (spherical to lenticular) cells.

Only a limited number of reports are related to flow cytometry with prokaryotic cells (4, 11, 12). Therefore, it was necessary to optimize several critical parameters for the concomitant analysis of two bacterial species. Above all, we show here that a fast and reliable method, based solely on light scattering at different angles, allows visualization in real time of individual populations of bacteria within a mixed culture. Classical microbiological methods were also used to validate this approach.

A mixed culture of *L. mesenteroides* Y105 (Bac^+ or Bac^-) and *L. monocytogenes* E20 was started, with listeriae in large excess (2×10^8 cells per ml) over the *Leuconostoc* cells (1.9×10^7/ml). *L. mesenteroides* Y105 (Bac^+) was isolated from goat milk (6), and *L. monocytogenes* E20 (serotype 4) belongs to the collection of the Institut Universitaire de Technologie, La Rochelle, France. A *Leuconostoc* mutant that does not secrete mesentericin Y105 (Bac^-) was isolated by repeated treatment with novobiocin (6 μg/ml), an antibiotic known to cure strains of plasmids (9). The strains were first grown to the late exponential phase at 30°C without agitation in tryptic soy broth (TSB) for *L. monocytogenes* and Man, Rogosa and Sharpe (MRS) broth for *L. mesenteroides* (Difco).

The individual behavior of the two strains was monitored by withdrawing a sample from the culture medium every 30 min for 6 h and analyzing it immediately by flow cytometry with an ACR 1500 (Bruker). The different strains were distinguished by light diffusion measurements (excitation filter, 475 ± 15 nm). Forward-angle scatter and right-angle scatter measurements reflect morphological characteristics (size, refringence, etc.). Optical parameters were determined by logarithmic amplification, with a flow rate of 2×10^3 cells per s. The flow rate was adjusted to count a maximal percentage (approximately 40%) of the cell population without enumerating cell debris. Under these conditions, each event (particle detected by the cytometer) represented an intact cell.

Data obtained at 0, 2, 4, and 6 h are displayed in Fig. 1. One would expect domination by *L. monocytogenes* at least at the beginning of the experiment, since the balance was largely in favor of this species; it represented ca. 90% of the total bacteria (8.8×10^7 versus 7×10^6 events per ml for *L. mesenteroides*), and the culture medium chosen (TSB) was optimized for *Listeria* growth, whereas *Leuconostoc* growth, which requires enriched medium (MRS or M17), should have been impaired. Figure 1A shows that in spite of those unfavorable conditions, the relative number of *Leuconostoc* (Bac^+) cells increased after 2 h, this strain becoming dominant after 4 h and finally being in large excess after 6 h (7.5×10^7 versus 4.6×10^7 events per ml for *L. monocytogenes*).

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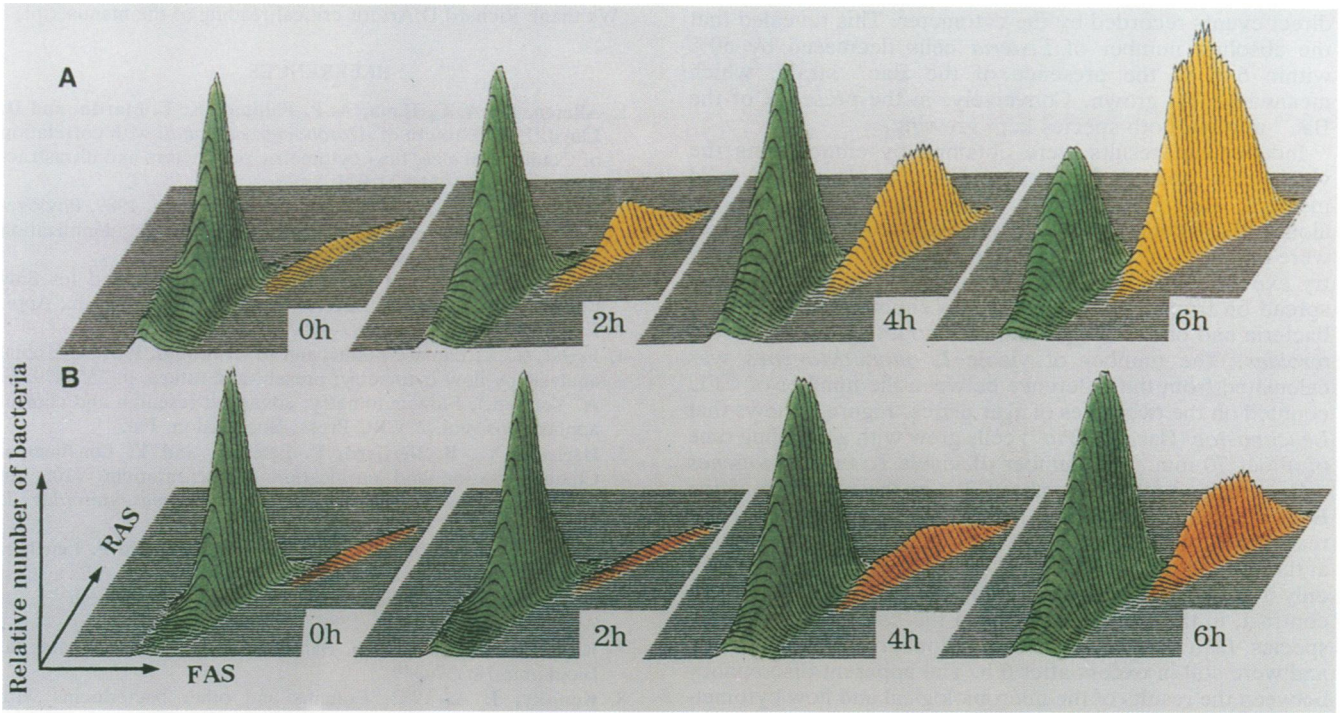

FIG. 1. On-line analysis by flow cytometry of the simultaneous growth behavior of *L. monocytogenes* E20 and *L. mesenteroides* Y105 (Bac^+) (A) or (Bac^-) (B) in a mixed culture. In the three-dimensional plots, *Listeria* cells (short rods, 0.4 to 0.5 μm in diameter, 0.5 to 2 μm in length) appear in green, whereas yellow and orange correspond to the *Leuconostoc* Bac^+ and Bac^- cells, respectively (lenticular, 0.5 to 0.7 μm in diameter, 0.7 to 1.2 μm in length). FAS, forward-angle scatter; RAS, right-angle scatter.

A control experiment performed under similar conditions with a *L. mesenteroides* Y105 mutant (Bac^-) showed quite a different effect (Fig. 1B). The relative number of *Leuconostoc* (Bac^-) cells increased but at a much slower rate than the wild-type cells, and *L. monocytogenes* was still the predominant strain at 6 h (7.6×10^7 and 1.4×10^8 events per ml, respectively). The relative decline in the number of *L. monocytogenes* cells could be due in both cases to growth arrest, related or not to cell death and lysis, or simply to a difference in the generation time of the two competing species. Complementary data were thus necessary to reach a conclusion. First, the number of cells was estimated from

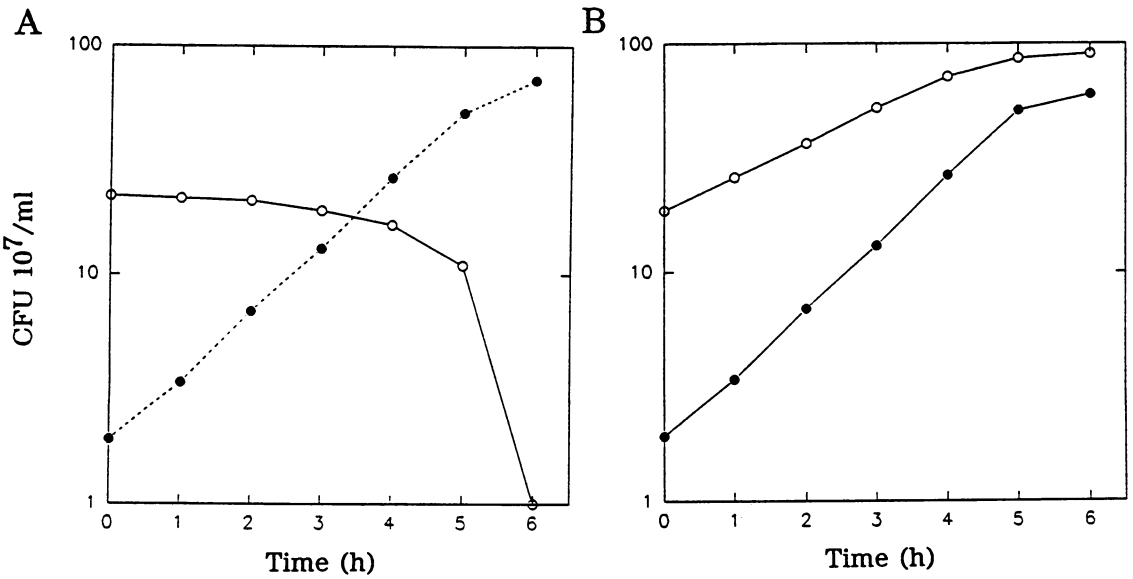

FIG. 2. Enumeration on agar plates of viable *L. monocytogenes* E20 cells (○) in a mixed culture with *L. mesenteroides* Y105 Bac^+ (A) or Bac^- (B) cells (●).

direct events recorded by the cytometer. This revealed that the absolute number of *Listeria* cells decreased by 50% within 6 h in the presence of the Bac^+ strain, which meanwhile had grown. Conversely, in the presence of the Bac^- mutant, both species kept growing.

Independent results were obtained by enumerating the viable cells able to form colonies on two types of solid medium, allowing growth of either the *Leuconostoc* sp. alone or both the *Listeria* and *Leuconostoc* species. Cultures were prepared and mixed as described for the flow cytometry experiments. At 30-min intervals, diluted samples were spread on tryptic soy agar plates to enumerate total viable bacteria and on MRS agar plates to count viable *L. mesenteroides*. The number of viable *L. monocytogenes* was calculated from the difference between the number of CFU counted on the two types of agar plates. Figure 2 shows that *Leuconostoc* (Bac^+ or Bac^-) cells grew with a doubling time of about 70 min. The number of viable *L. monocytogenes* cells remained stable for about 2 h in the presence of the Bac^+ strain and then tended to decline dramatically after 5 h, reaching a level of less than 10^7 cells per ml at 6 h. Therefore, at the end of this experiment, *L. monocytogenes* represented only 1 to 2% of the overall viable bacterial population. In contrast, in the mixed culture with the *Leuconostoc* (Bac^-) species, *Listeria* cells grew with a doubling time of 110 min and were still in excess after 6 h. The apparent discrepancy between the results of the microbiological and flow cytometric approaches comes from the facts that the flow rate chosen was too high to visualize all of the bacterial cells and that only physical parameters were analyzed with the latter method. Therefore, at 6 h, the *Listeria* cells visualized in Fig. 1A represented mainly dead but apparently intact bacteria.

It can be concluded from this study that *L. mesenteroides* Y105 (Bac^+) exerts a bactericidal effect against *L. monocytogenes*, leading to cell lysis. Flow cytometry appears to be a fast and reliable method for demonstrating the efficiency of strains producing antimicrobial compounds. Moreover, it will hopefully become a convenient tool for detecting specific bacterial species by using selective immunofluorescent (3, 4, 11) or rRNA-based probes (2). This method thus seems particularly suited for following, on line and in real time, the simultaneous development not only of antagonistic strains but also, more generally, of mixed cultures of bacteria.

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