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Growth and Survival of *Serratia marcescens* under Aerobic and Anaerobic Conditions in the Presence of Materials from Blood Bags

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Several patients receiving blood transfusions during the summer of 1991 developed bacteremia after the transfusion. In all cases, the infection was caused by *Serratia marcescens*. The same strain of *Serratia marcescens* was isolated from the patients and from the outer surface of unfilled blood bags. The transport containers for the blood bags were made anoxic by using a catalyst in order to prevent microbial growth. The survival and growth of *S. marcescens* K202, which was isolated from the blood bags, was studied at different oxygen concentrations in deionized water containing materials derived from the blood bags. The rate of survival and growth of *S. marcescens* was highest under anaerobic conditions, in which growth occurred with all materials and even in deionized water alone. In contrast, *S. marcescens* did not survive in control cultures under semi-anaerobic and aerobic conditions. Growth was observed, however, under both aerobic and semi-anaerobic conditions in the presence of each of the tested blood bag materials. These findings indicate that the conditions in the transport containers for the blood bags were favorable for the survival and growth of *S. marcescens*.

The gram-negative enterobacterium *Serratia marcescens* is commonly found in soil and water but also inhabits man-made environments. For a long time, *S. marcescens* was thought to be a harmless environmental organism but is now considered to be an opportunistic pathogen. *S. marcescens* causes nosocomial infections associated with various kinds of medical equipments, such as mechanical respirators, intravenous catheters, and ultrasonic nebulizers, and with intravenous fluids (4, 24).

The widespread occurrence of *S. marcescens* indicates a strong adaptive and survival potential and the ability to utilize a wide range of nutrients. This potential is clearly expressed by the ability of *S. marcescens* to survive and grow under extreme conditions, including in disinfectant solutions (13, 19), antiseptics (18), and double-distilled water (1). The bacterial cells readily colonize surfaces and are enriched at the air-water interface (9, 22), most probably because of the pronounced cell surface hydrophobicity of *S. marcescens*. In addition, it was shown that *S. marcescens* is able to utilize surface-bound nutrients, such as long chain fatty acids (10).

An outbreak of nosocomial infections during the summer of 1991 in Sweden and Denmark was confirmed to be attributable to *S. marcescens*, and the incidences correlated with blood transfusions. This prompted questions about the source of the organisms. In a separate study, it was demonstrated that *S. marcescens* could not be isolated from the inside of unfilled blood bags; however, *S. marcescens* could be isolated in several cases from the outer surfaces of blood bags, originally packed in the transport containers (1a). By using biotyping and ribotyping for comparison of the patient isolates and the strains isolated from the blood bags, it was noted that the blood bags must have been the source of the infections.

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During the assembly of the blood bags in the factory, the bags were autoclaved and then cooled with (unsterile) deionized water. The bags were then manually packed into the transport containers, resulting in carryover of the cooling water into these containers. A catalyst was added to the containers to create microaerophilic conditions.

The aim of this study was to examine how *S. marcescens* can grow and survive under the conditions present in the transport containers. Special attention was paid to the influence on growth and survival of substances originating from material from the blood bags and the partial pressure of oxygen.

MATERIALS AND METHODS

Bacteria and growth conditions. *S. marcescens* K202 was isolated from the outside of unfilled blood bags at the National Bacteriological Laboratory (1a) and was used for all examinations described in this paper. *S. marcescens* was cultivated in tryptic soy broth (TSB; Difco). For survival tests, *S. marcescens* was grown in TSB over night and then centrifuged, and the pellet was washed twice with phosphate-buffered saline (PBS) prior to final resuspension in PBS to an optical density of 0.1 (λ = 600 nm). This corresponded to a cell density of 10^8 CFU/ml. This bacterial cell suspension was further diluted 1:10,000 and used in all experiments by inoculating to the desired final cell density. Cell counts were carried out with mEndo Agar LES (Difco).

Survival studies of *S. marcescens* in the presence of material pieces. *S. marcescens* was incubated in deionized water in the presence of pieces of materials from the blood bags and the transport box. Pieces of the following materials were used: outer transport box (M1), paper from the bottom of the transport box (M2), unfilled blood bag without label (M3) and with label (M4), tubing connecting the bags (M5), and blood bag containing CPD solution (glucose and citric acid in phosphate buffer) (M6) or SAGM solution (glucose, manni-

TABLE 1. Number of *S. marcescens* cells after incubation in nonautoclaved or autoclaved deionized water alone (control) or in the presence of different blood bag materials (M1 to M7) under different incubation conditions[a]

Material	No. of cells (CFU/ml) at:					
	37°C for 4 days				20°C for 6 days	
	Nonautoclaved		Autoclaved		Autoclaved	
	Aerobic	Semi-anaerobic	Aerobic	Semi-anaerobic	Aerobic	Semi-anaerobic
Control	<10	50	<10	7.1×10^5	<10	$5.0 \times 10^5 \pm 4.6 \times 10^5$
M1	1.1×10^6	1.1×10^6	2.9×10^5	8.4×10^5	$2.4 \times 10^5 \pm 2.0 \times 10^5$	$1.4 \times 10^5 \pm 2.5 \times 10^5$
M2	2.7×10^6	1.6×10^6	1.9×10^7	1.2×10^7	$2.6 \times 10^6 \pm 0.3 \times 10^6$	$1.3 \times 10^6 \pm 0.7 \times 10^6$
M3	1.5×10^4	2.1×10^4	8.2×10^6	1.0×10^6	$1.9 \times 10^5 \pm 1.7 \times 10^5$	2.1×10^5
M4	2.6×10^5	1.8×10^5	3.0×10^7	3.0×10^7	$3.0 \times 10^7 \pm 1.8 \times 10^7$	$1.6 \times 10^6 \pm 0.5 \times 10^6$
M5	1.2×10^5	1.9×10^5	3.0×10^7	1.2×10^7	$1.2 \times 10^6 \pm 0.2 \times 10^6$	$2.1 \times 10^5 \pm 3.7 \times 10^5$
M6	6.4×10^5	1.2×10^5	1.7×10^7	1.3×10^6	ND	ND
M7	8.5×10^5	3.7×10^5	7.0×10^6	2.7×10^6	ND	ND

[a] The initial number of cells at day 0 was 10^2 CFU/ml. The experiments at 37°C were done in duplicate; the values given represent the mean values. The experiments at 20°C were done in triplicate; the mean values ± the standard deviations are given. ND, not determined.

tol, and adenine in a sodium chloride solution) (M7). The materials were cut into pieces, placed in glass tubes, and covered with deionized water. Half of each preparation was autoclaved at 120°C for 20 min, except for material M1, which was boiled for only 5 min because it was not resistant to autoclaving. The other half was left unsterilized. Tubes with autoclaved or nonautoclaved deionized water alone served as controls. Cells of *S. marcescens* were added to all tubes to a final density of 100 CFU/ml. Half of the autoclaved and nonautoclaved tubes of each material were incubated in anaerobic jars (referred to as semi-anaerobic), while the other half was incubated aerobically. This resulted in four different incubation conditions for each material. All tubes were incubated at 37°C. After 4 days, the tubes were vortexed for 30 s, and samples were taken for the determination of the number of culturable cells. The experiments were carried out in duplicate.

Another set of tubes with materials M1 to M6 in autoclaved deionized water was treated in a similar way but incubated at 20°C to better mimic the conditions during storage of the blood bags. Samples were taken after 6 days of incubation. These experiments were carried out in triplicate.

Survival studies with extracts from different materials. *S. marcescens* was incubated in aqueous extracts of several materials. Pieces of materials M2 to M5 were used for extractions. The pieces of material were covered with deionized water and autoclaved for 20 min (120°C). Half of the supernatant of each material was cooled under a gas mixture of N_2 and CO_2 (90%:10% [vol/vol]) (anoxic extract); the other half was cooled under air (oxic extract). At the start of an experiment, aliquots of the respective extracts were transferred to glass tubes.

Three different incubation conditions were used to test the survival of *S. marcescens* in aqueous extracts of the different materials. These conditions included aerobic and semi-anaerobic (anaerobic jar) conditions as well as strictly anaerobic conditions (Hungate technique [2]) The experiments under aerobic conditions were carried out with the oxic extracts and glass tubes with polyethene caps. The anoxic extracts were transferred either to the polyethene-capped tubes or to pregassed tubes closed with butyl rubber stoppers for semi-anaerobic or strictly anaerobic conditions, respectively. Tubes with autoclaved deionized water alone served as controls and were treated in a way similar to that for the extracts to obtain the different incubation conditions. In all cases, care was taken to prevent any contact between the extract and the caps or the stoppers to avoid contaminations from these plastic materials. Aliquots of a cell suspension of *S. marcescens* were added to each tube in order to obtain a final density of 100 CFU/ml. The tubes containing anoxic extracts or anoxic water were then either transferred to anaerobic jars for semi-anaerobic conditions or gassed with N_2-CO_2 for strictly anaerobic conditions. The tubes containing oxic extracts or oxic water were incubated in air without shaking. All experiments were carried out at least in triplicate at 37°C.

The number of cells in the various extracts and controls was determined after different time intervals. Samples from both the aerobic and semi-anaerobic tubes were always taken from the same tubes. By contrast, many parallel tubes were included for the strictly anaerobic conditions. A new set of tubes was used for each sampling to assure oxygen-free conditions during the whole time of incubation.

RESULTS

Survival and growth of *S. marcescens* in the presence of pieces from blood bags. *S. marcescens* was introduced to the test tubes at cell densities of 100 CFU/ml and incubated either aerobically or semi-anaerobically. After 4 days, the number of *S. marcescens* cells had increased in all tubes, except for the aerobic controls in which no cells could be cultured. In the nonautoclaved anaerobic control, very few bacteria were culturable. The increase in the number of cells in the other test tubes was between 10^2- and 10^5-fold (Table 1). *S. marcescens* grew better in the autoclaved samples than in the nonautoclaved parallels. This was true for all materials, except material M1, and for the semi-anaerobic controls.

Comparable results were obtained with autoclaved deionized water at an incubation temperature of 20°C (Table 1). The number of cells obtained after 6 days of incubation was in most cases lower than that obtained at 37°C after 4 days.

Survival of *S. marcescens* in aqueous extracts from materials from blood bags. In addition to the aerobic and semi-anaerobic conditions used in the previous experiment, strictly anaerobic conditions were included in these experiments with aqueous material extracts. The degree of survival and growth of *S. marcescens* was influenced by the concentration of oxygen and the type of material used for the extraction.

S. marcescens was not detectable in the aerobic control after 4 days (Fig. 1a). In the tubes containing extracts from

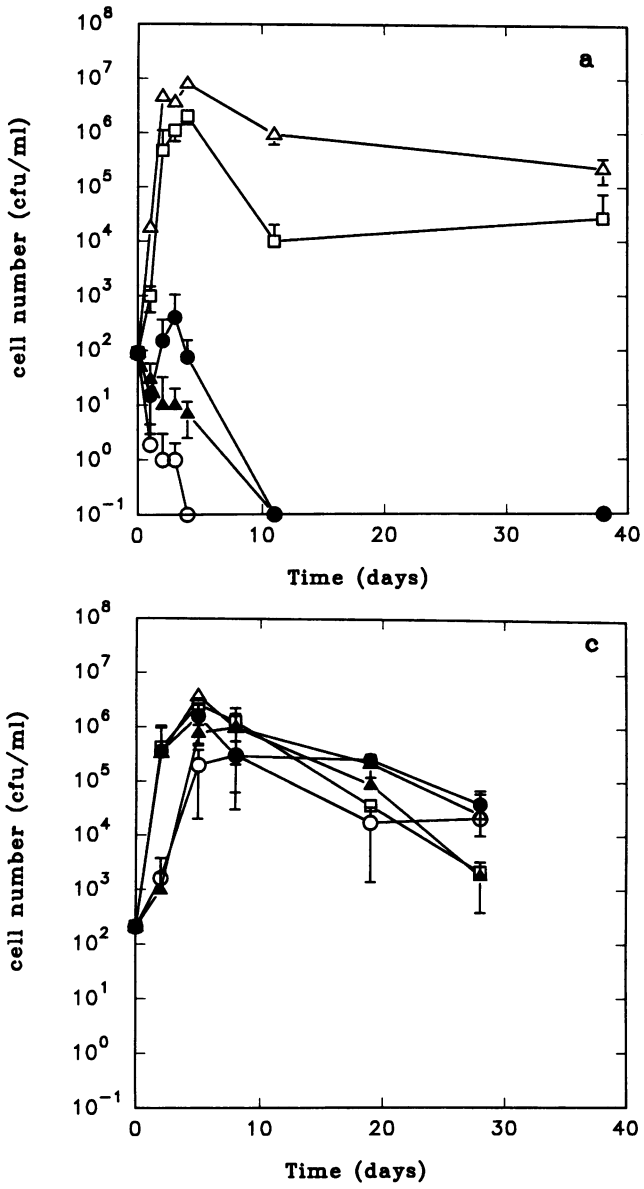

FIG. 1. Quantification of *S. marcescens* in autoclaved deionized water (○) and in aqueous extracts of material M2 (□), M3 (▲), M4 (△), and M5 (●) under aerobic (a), semi-anaerobic (b), or strictly anaerobic (c) incubation conditions for up to 38 days. Mean values are shown; error bars indicate standard deviations.

paper or plastic with a label, the number of cells increased 10^4- to 10^5-fold during the first 5 days and then slowly decreased. After 28 days, the numbers were still 10^3- to 10^4-fold higher than at the onset of the experiment. *S. marcescens* survived better in the extracts from only plastic materials (tubing and bag) than in the control; however, no significant increase in the number of cells was observed. After 11 days, no cells could be cultured from these tubes (Fig. 1a).

S. marcescens grew and survived in all semi-anaerobic extracts (Fig. 1b). The highest growth yield was observed in the extracts from materials containing paper. In the control, *S. marcescens* showed fluctuations in the number of cells during the first 10 to 15 days. The standard deviation was high for these experiments because of different survival times of the bacteria in the parallel cultures. This was probably due to variations in exposure to oxygen during sampling.

Under strictly anaerobic conditions, *S. marcescens* grew and survived in all parallels including the control (Fig. 1c). The maximum number of cells was approximately 10-fold higher in the extracts than in the control. The maximum number of cells was reached after approximately 5 days, after which time the number of cells slowly decreased. After 28 days, the number of cells was still 10- to 100-fold higher than the number at beginning of the experiment (Fig. 1c).

DISCUSSION

The survival and growth potential of *S. marcescens* in the presence and absence of materials from blood bags were examined under aerobic, semi-anaerobic, and strictly anaerobic conditions. The behavior of *S. marcescens* in autoclaved deionized water was dependent on the oxygen partial pressure in the test tube. *S. marcescens* did not survive for more than 4 days under aerobic conditions. Survival of *S. marcescens* was much better under semi-anaerobic conditions in which the number of cells slowly decreased, and after approximately 20 days no cells were culturable. Under strictly anaerobic conditions, *S. marcescens* grew several-fold in the control, probably because of residues from the indigenous microflora which was killed during autoclaving. These results clearly show a toxic effect of oxygen on *S. marcescens* in low-nutrient conditions.

During the last decade, the survival of bacteria under starvation conditions has been studied extensively for marine bacteria (8, 11, 12, 17) and in fresh water (3, 15, 16). These studies have also included members of the family *Enterobacteriaceae* (3, 21, 23) and have revealed a high degree of survival for many bacteria. Most of these starvation experiments were carried out with high initial bacterial cell densities (10^6 to 10^8 cells per ml) compared with that used in our experiments (10^2 cells per ml). The influence of the oxygen partial pressure on the survival of the bacteria has been neglected in all these starvation experiments. An initially high number of cells may lead to better survival of

the bacteria not only by cryptic growth (14) but also by reducing the oxygen partial pressure in the suspension because of respiration of the cells, thereby eliminating the toxic effect of oxygen radicals. In a recent study, it was demonstrated that inactivation of oxygen radicals enhanced survival of *Escherichia coli* (7). Botzenhart and Kufferath used an initially low number of cells and demonstrated that *S. marcescens* grew under aerobic conditions in double-distilled water as well as deionized water (1). The strain of *S. marcescens* used by these investigators was obviously less oxygen sensitive than our strain K202.

The presence of blood bag materials promoted growth of *S. marcescens* under all incubation conditions. Growth at 37°C was better in the autoclaved parallels than in the nonautoclaved parallels, except for the tests with material M1. Two explanations are possible for these results. Firstly, because of competition with or inhibition by the indigenous flora from the deionized water, the number of *S. marcescens* cells was lower than the number in the experiments with autoclaved deionized water. Secondly, more substances could have leached out from the different materials because of the autoclaving and were therefore available to the bacteria. The first explanation can be excluded because *S. marcescens* was the dominating bacterium in the nonautoclaved tubes after 4 days of incubation and very few other bacteria were detected, indicating that the indigenous flora was overgrown by *S. marcescens* (data not shown). Further support for the second explanation is the comparable growth of *S. marcescens* on material M1 under sterile and unsterile conditions, because this was the only material not autoclaved.

The results from the experiments with aqueous extracts of the materials also showed that substances leaching out of the blood bag materials during autoclaving promoted growth of *S. marcescens*. *S. marcescens* grew in the extracts from all materials under semi-anaerobic and strictly anaerobic conditions, while under aerobic conditions the extracts from only the paper and the labeled blood bags supported growth. Leaching of nutrients from the blood bags similar to that which we simulated in our experiments may have happened in the production process when autoclaved hot bags were cooled with deionized water. The results again show the importance not only of the oxygen concentration but also of the type of nutrients leaching out of the materials. These latter findings are in agreement with those of other studies demonstrating that extremely low concentrations of organic material can be sufficient not only for survival but also for active growth of bacteria (1, 5, 20). Furthermore, the introduction of organic molecules from the air, e.g., solvents, has been shown to be sufficient to support growth of certain bacteria (6). Substances other than carbon sources, i.e., phosphates, vitamins, or trace elements, may enhance survival or promote growth under low-nutrient conditions (1).

Our studies were initiated to examine how the opportunistic pathogen *S. marcescens* could survive and grow under semi-anaerobic conditions in the transport containers for blood bags. From the results, it is clear that both the presence of moist materials from blood bags or of extracted compounds from these materials and the reduced oxygen partial pressure supported growth of *S. marcescens* K202. Currently, experiments are being carried out in our laboratory to determine the nature of the substances leaching out of the blood bag materials and promoting growth of *S. marcescens*.

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