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RESEARCH ARTICLE

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Identification of synergistic interactions among microorganisms in biofilms by digital image analysis

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Summary Digital image analysis showed that reductions in biofilm plating efficiency were due to the loss of protection provided by two benzoate-degrading strains of *Pseudomonas fluorescens*. This loss in protection was due to the spatial separation of the protective organisms from benzoate-sensitive organisms during the dilution process. Communities were cultivated in flow cells irrigated with trypticase soy broth. When the effluent from these flow cells was plated on 0.15% benzoic acid, satellite colonies formed only in the vicinity of primary colonies. A digital image analysis procedure was developed to measure the size and spatial distribution of these satellites as a function of distance from the primary colony. The size of satellites served as a measure of growth, and the number per unit area served as a measure of survival. At the three dilutions tested, the size and concentration of satellite colonies varied inversely with distance from the primary colonies. When these measurements were plotted, the slopes were used to quantify the effect of bacterial association on the growth and survivability of the satellites. In the absence of the primary colonies, satellites grew in axenic culture only at low benzoate concentrations. Thus benzoate-degrading organisms are capable of creating a protective microenvironment for other members of biofilm communities.

Key words Satellite colonies · Protective interactions · Food preservation · Sodium benzoate · Synergism

Introduction

Community-level synergisms are required for the optimal proliferation of biofilm communities, just as molecular synergisms are required to optimize the proliferation of cells [6–9]. One indication of these synergisms is the density-dependence of plating efficiency that sometimes occurs when isolating or enumerating microorganisms using plating media. This was first noted by Stevens and Holbert [Abstracts of the Conference on Multicellular and Interactive Behavior of Bacteria, ASM. MBL, Woods Hole, 1990, p. 20] when plating bacterial communities from subsurface terrestrial environments. Caldwell et al. [6] subsequently observed increased plating efficiency at lower dilutions only when plating microbial communities at high concentrations of benzoate, but not at low concentrations. In the present study digital image analysis was used to examine the effect of spatial positioning on the growth and survival of colonies during the plating process. Sodium benzoate was used in these studies due to its importance as a model compound in the degradation of organic toxicants and in the preservation of various food products [17, 21]. Benzoate is also significant due to its inhibitory effect on the bacteria which cause dental plaque [20], food spoilage [12, 26], and foodborne illness [17].

The results showed that during the plating process the primary benzoate-degrading organisms of the community protected the other organisms with which they were associated.

The protected organisms appeared as satellite colonies located within the vicinity of the primary benzoate-degrading organisms. The irregular spacing of both primary colonies and satellite colonies can sometimes obscure the satellite effect.

Materials and methods

Bacterial communities, strains and media A composite inoculum was prepared by mixing 0.5 g each of two oil-sludge samples (Kamsack and Baildon sites, Saskatchewan, Canada), a pristine soil sample (Matador grasslands, Saskatchewan, Canada) and a pesticide disposal-pit soil sample (Iowa State University, Iowa, USA), in 50 ml of mineral salts solution [27]. This was used to inoculate a flow cell [18] (channel dimensions: 1 mm by 3 mm by 40 mm) continuously irrigated with 0.3% (w/v) trypticase soy broth (TSB) (Sigma Chemical Co., St. Louis, MO) at a flow rate of 0.1 mm/s. This TSB community reached a quasi-steady state in 100 h. The quasi-steady state was determined based on the stability of biofilm percent area coverage and thickness (monitored by scanning confocal laser microscopy at 12 h intervals) and based on the consistency of colony morphotypes which appeared when the effluent was plated on 0.3% (w/v) trypticase soy agar (TSA).

The satellite isolates described in this study were maintained on TSA and the primary colony isolates were maintained on benzoate agar (BA) described below. Two bacterial strains MPT1 and MPT2, which were different from all the primary and satellite isolates (based on colony morphotypes on 0.3% (w/v) TSA), were isolated from the TSB community and maintained on TSA. These two bacterial strains, as well as a culture collection strain of *Escherichia coli* (ATCC 25922) were used in the specificity studies.

Benzoate agar (BA) and glucose agar (GA), were prepared by addition of appropriate carbon sources to the minimal-salts agar. Unless specified otherwise, BA contained 0.15% (w/v) benzoic acid (sodium salt) (Sigma Chemical Co., St. Louis, MO) and GA contained 0.1% (w/v) glucose (BDH Inc., Toronto, Canada). The BA plates were poured using an equal quantity of the medium in each Petri dish. This was to prevent any volume-dependent differences in the concentration of growth factors or degradation products that might be released by the primary colonies into BA.

Plating assays This study involved various plating procedures including an initial assay for dilution-dependent plating efficiency of the TSB community on BA (assay i, shown below). When satellite colonies were observed on BA plates, putative satellite and primary colonies were isolated. Cross-streak (assay ii) and spot inoculation assays (assay iii) were used to confirm association between individual primary and satellite isolates. Additional assays were used to examine synergisms based on interactions among multiple primary and satellite isolates (assay iv), to quantify interactions between primary and satellite isolates (assays v, vi and vii), to identify the mechanisms of their association (assay viii) and to examine the specificity of the associations (assay ix). Preparation of standard cell suspensions for various plating assays involved growing cells to log phase in 0.3% (w/v) TSB, centrifugation at 7800× *g* for 10 min followed by resuspension of cells in physiological saline (0.85%) and adjusting to a turbidity standard to give an approximate cell number of 10^8 CFU/ml. Plate incubations were performed at 24°C for 7 days.

i. Determination of dilution-dependent and propinquity-dependent plating efficiency Ten ml of flow-cell effluent was collected over a 5 h period, from the flow-cell containing the TSB community. The effluent was serially diluted and plated on BA medium. The dilution scheme consisted of ten-fold dilutions (1×10^{-1}, 1×10^{-2}, 1×10^{-3}, etc.) and subdilutions between two major dilutions (e.g. from 0.1×10^{-2}, 0.2×10^{-2}... to 0.9×10^{-2}). The plates were incubated and the plating efficiency (CFU/ml) was determined at various dilutions. Determination of propinquity-dependent plating efficiency involved calculation of mean separation distance between cells when they were plated at various dilutions. A procedure similar to that of Thomas et al. [Abstracts of the Conference on Multicellular and Interactive Behavior of Bacteria, ASM. MBL, Woods Hole, 1990, p. 20] was used for this purpose. The number of cells inoculated per plate (n) at various dilutions was calculated from the cell number in the inoculum (maximum CFU/ml was used in this case) and the corresponding dilution factor. Area of the plate divided by n gives the mean area available to a single cell. Approximating this area to that of a circle, the radius of each circle was determined. Twice this radius was the mean cell separation distance. The cell separation distances (as a measure of propinquity) were then correlated with the plating efficiency.

ii. Cross-streak assay Cell suspensions of putative satellites and primary colonies were streaked perpendicularly against each other on BA plates. After incubation for 7 days, the growth patterns of the primary and satellite strains were observed and documented.

iii. Spot-inoculation assay The spot inoculation assay was performed by serially diluting the putative satellites and spreading them on BA plates before spotting a 5 µl aliquot of a primary isolate cell suspension at the center. After incubation, plates were observed for the appearance of satellite colonies around the spot of primary isolate.

iv. Assay for the detection of synergistic effects involving three or more organisms The primary isolates (BD1 and BD2) were streaked on 0.15% BA containing a lawn of a satellite strain and a second satellite isolate was then streaked perpendicular to BD1 and BD2. Plates were incubated for 7 days and examined for synergistic growth effects resulting from interactions among three or more members.

v. Quantitative analysis of interactions between satellite and primary colonies Appropriate dilutions of the standard

cell suspensions of satellite isolates were spread as a lawn on BA plates (each plate received approximately 10^3 cells). A 2 µl aliquot of standard cell suspension of a primary colony was then spot-inoculated at the center of the plate. Satellite colonies appearing around primary colonies were analyzed using the digital image analysis procedure described below. When the spatial distribution of the satellites was plotted with respect to the location of primary colonies, the slopes were calculated from peak of the curves to the right (Fig. 4B and D). This was necessary because of the reduction in the number of satellites/cm^2 adjacent to the primary colony due to the sharing of limiting substrate concentration. Including this effect in the calculation would mask the satellite effect (dependency of satellites on primary colony) quantified from the slope.

vi. Analysis of the effect of satellite cell concentration on primary colony–satellite interactions Three 10-fold dilutions of a standard cell suspension of the satellite isolate BS2 were spread in triplicate on plates containing BA (at satellite concentrations of 2.2×10^4, 2.2×10^3, 2.2×10^2 satellites/cm^2, respectively). Two µl of the standard cell suspension of a primary colony (BD2) was then spot-inoculated at the center of satellite lawns, plates were incubated and analyzed using the digital image analysis procedure.

vii. Analysis of the influence of satellites on primary colonies Satellite and primary isolates were spotted on BA plates in the form of a rectangular 3×3 matrix. Three spots of primary isolate (aliquots of 2 µl each) formed the central row, whereas a suspension of the satellite isolate (aliquots of 2 µl each) was spotted in top and bottom rows. Following incubation, the growth of primary and satellite isolates was digitized as described in the image acquisition part of the digital image analysis procedure. Subsequently, the sizes of primary colony spots were determined using the following procedure: NIH image software (National Institutes of Health, Bethesda; http://rsb.info.nih.gov/nih-image) was used to invert and to redefine the image by setting a threshold. The mean size of primary colonies (BD1 and BD2), with and without adjacent spot inoculation of satellites was then determined using the particle analysis function of NIH image.

viii. Analysis of the mechanism of satellite associations Serial dilutions of satellite cell suspensions were spread-plated on BA with 9 different concentrations (0.015%, 0.05%, 0.1%, 0.15%, 0.2%, 0.25%, 0.5%, 1.0%, 1.5%) of benzoic acid, with and without added glucose (1 g/l). Primary isolates were then spot-inoculated at the center of satellite lawns. Controls with no inoculation of primary isolate were also maintained. Growth of satellite colonies on these plates was then compared with the growth on GA (glucose as sole carbon source). In another set of experiments, agar plugs of 10 mm diameter were removed from areas adjacent to 7 day old, spot-growth of primary isolates, and placed on 0.15% BA plates previously inoculated with a lawn of satellite colonies. The plates were examined for the development of satellite colonies around agar plugs. 10 µl aliquots of homogenates obtained by homogenizing an agar plug with 1 ml quantity of mineral salts solution were also used to spot on lawns of satellite isolates and after 7 days incubation, the plates were examined for the development of satellite colonies around the spots.

ix. Analysis of the specificity of bacterial interactions The specificity of the primary colony-satellite associations was tested by cross streak assays on BA and BA supplemented with glucose (1 g/l). The standard cell suspensions of the primary colonies BD1 and BD2 were streaked perpendicularly against standard cell suspensions of *E. coli* (ATCC 25922) and two morphotypes (MPT1 and MPT2) derived from the TSB community. The plates were incubated and examined.

Digital image analysis of primary colony-satellite interactions Analysis of size and spatial distribution of satellite colonies around primary colonies involved image acquisition and digital image analysis.

i. Image acquisition The satellite associations which developed on BA plates were digitized using a Scion video board and NIH (National Institute of Health) image software in conjunction with a Tamron SP-CF macro lens (Tamron Co. Ltd., Japan) mounted on a Cohu high performance CCD camera (Cohu Inc., Electronics Division, San Diego, CA). The lens was stopped down (F32) to reduce spherical aberration and to increase the depth of field. The Petri dish with satellite colonies to be digitized was placed on top of an inverted Petri dish containing agar (1.5% w/v) with 0.12% (w/v) brilliant black food colorant (Sigma Chemical Company, St. Louis, MO) in order to reduce the background brightness to as close to zero as possible. The electronic gain was manually set from within the NIH image software to produce high contrast images for analysis purposes. For each plate, 20 images were digitized at video rate and averaged to minimize electronic interference.

ii. Digital image analysis Analysis of the size and number of satellites, as a function of the distance from the primary colony, involved the use of Superpaint (Silicon Beach Software, Inc. San Diego, CA), Adobe Photoshop (Adobe Systems, Inc., Mountain View, CA), and NIH image applications. A mask of nested circular rings with diameters ranging from 42 pixels to 462 pixels was created using Superpaint. The camera height and magnification were set such that the width of each analysis ring corresponded to a distance of 1.5 mm (a distance of 21 pixels). This mask was used to create a template in Adobe Photoshop with each ring in a separate layer, so that if one layer was made transparent the colonies in the image appearing through the transparent ring could be analyzed. For analysis, the image showing the transparent ring with colonies was copied to NIH image, inverted, thresholded and the number of colonies and colony sizes were measured within each ring. Since each ring was placed at a known distance from the primary colony,

the number and size of satellites (within that ring) as a function of distance could be determined.

Characterization of primary and satellite colonies Primary and satellite isolates were streaked on 0.3% (w/v) trypticase soy agar and incubated at 24°C for 7 days. The morphotypes of isolated colonies were determined using the descriptions of Benson [1] and Eklund and Lankford [13]. Gram reaction and cell morphologies were determined using light microscopy. Tentative identification of the isolates was based on API-NFT (BioMérieux Vitek, Inc., Hazelwood, MO) identification profiles (following instructions of manufacturer) and confirmation was based on *Bergey's Manual of Determinative Bacteriology* [16]. This included the tests of motility, production of diffusible fluorescent pigments, oxygen requirement, oxidase and other biochemical characters (nitrate reduction, arginine dihydrolase, gelatinase, esculin hydrolase, urease activities, indole production, glucose fermentation) and assimilation of various sugars and organic acids (glucose, arabinose, mannose, mannitol, N-acetyl glucosamine, maltose, gluconic acid, capric acid, adipic acid, malate and citrate).

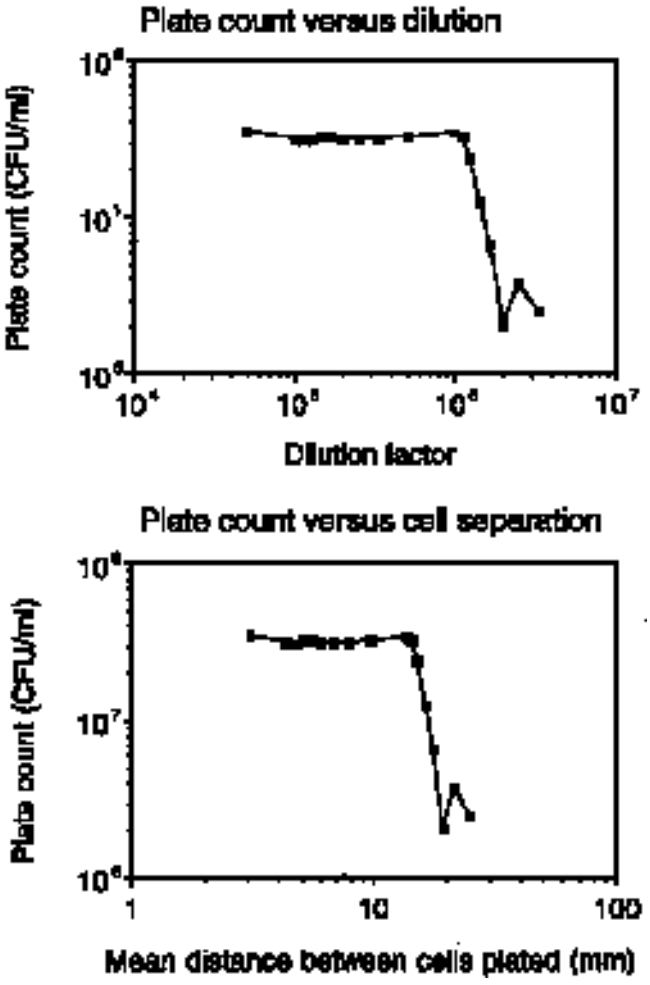

Fig. 1 Dilution-dependent plating efficiency observed when biofilm communities were plated on a synthetic medium containing 0.15% sodium benzoate as sole carbon source

Results

Effect of dilution on plating efficiency When the TSB community was plated on BA, there was an inverse relationship between plating efficiency and dilution (Fig. 1). The CFU/ml were higher at lower dilutions (at lower cell separation distances) and vice versa. A maximum CFU of $6.0 \times 10^6 \pm 5.5 \times 10^5$ SD/ml and a minimum of $3.7 \times 10^5 \pm 6.4 \times 10^4$ SD/ml were obtained for the same biofilm community at the various dilutions counted. CFU/ml of the bacterial community was maximum, when the mean distance between cells plated was less than 12 mm (Fig. 1).

Screening for satellite associations When the TSB community was plated at lower dilutions on BA plates, satellite colonies appeared around two types of primary colonies. When the total number of primary colonies on a plate was large, the satellite effect was diminished and the satellites tended to grow across the entire plate, as they did when the concentration of benzoate in the plating medium was reduced to less than 0.15%. The irregular distribution of both primary colonies and satellite colonies also tended to obscure the satellite effect, which was most clearly seen when a primary colony was inoculated upon a diffuse lawn of satellite colonies.

Each satellite was isolated and tested for interaction with the two primary colonies on 0.15% BA. Two associations were found, each involving a set of 7 satellite colonies. These were designated as the BD1 association (benzoate degrading association 1) and BD2 association, based on the primary colonies (strains BD1 and BD2) which allowed satellite colony development. Both primary colonies, BD1 and BD2 were identified as *Pseudomonas fluorescens* strains. The satellite colonies were *Pseudomonas* sp. strain BS1 (benzoate satellite 1), *Pseudomonas* sp. strain BS2, *Pseudomonas* sp. strain BS3, *P. fluorescens* strain BS4, *Pseudomonas viridiflava* strain BS5, *Pseudomonas* sp. strain BS6 and *Pseudomonas* sp. strain BS7. Both BD1 and BD2 supported the 7 satellites BS1, BS2, BS3, BS4, BS5, BS6 and BS7. Positive interaction between primary colonies and satellites of each association was confirmed by cross-streak and spot-inoculation assays, in which the growth of primary isolates promoted the growth of satellite strains. However, no synergistic growth effect due to interactions requiring three or more isolates could be detected. In addition, the satellites were unable to grow independently on 0.15% BA.

Quantification of satellite relationships The size and

distribution of the satellites were analyzed using digital image analysis. Within both sets of interaction (BD1 and BD2 associations), the satellite colonies decreased in size with distance from the primary colony. The mean satellite concentration (satellites/mm^2) also decreased with distance from the primary colony in both associations. Mean satellite concentrations were maximum, at a distance of approximately 7 mm from the center of the primary colonies. However, closer to the edge of the primary colony (3–7 mm from the center of the primary colonies), the number of satellites decreased with decreasing distance (Fig. 2).

The size and frequency of individual satellites was determined as a function of distance from the primary colony (Fig. 3). Each satellite showed a positive interaction with both primary colonies as indicated by the negative slopes of the size distribution and satellite concentration curves (Fig. 4). The slopes ranged from –0.0192 to –0.0893 (colony size in mm/distance from primary colony in mm) for size distribution and from –0.0242 to –0.0556 (number of satellites per mm^2/distance from primary colony in mm) for mean satellite concentration in the BD1 association (Fig. 4A, B). The values ranged from –0.0254 to –0.0801 for size distribution and from –0.0114 to –0.0490 for mean satellite concentration in the case of BD2 association (Fig. 4C, D). High correlation coefficients ($R^2 > 0.80$) were noted between the mean sizes of satellites and their distance from the primary colony in all the pairs analyzed.

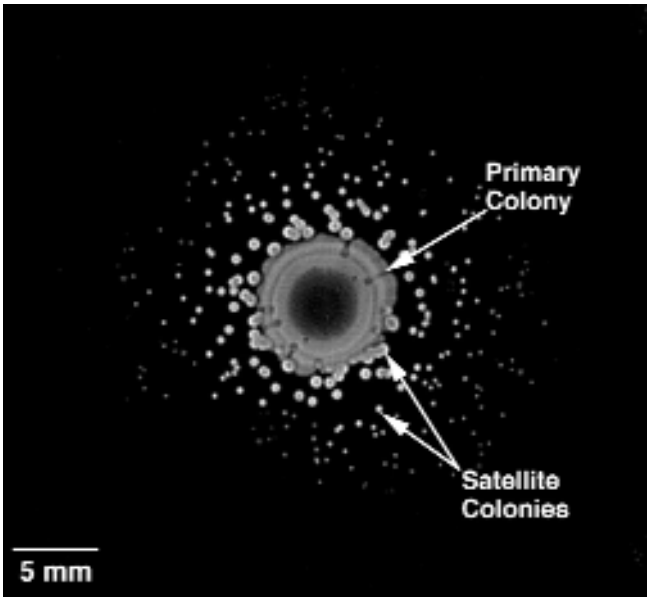

Fig. 3 An example of satellite plating procedure for the quantitative analysis of spatial interactions between individual primary and satellite colonies. Image shows BD1 spot-inoculated on a lawn of BS2

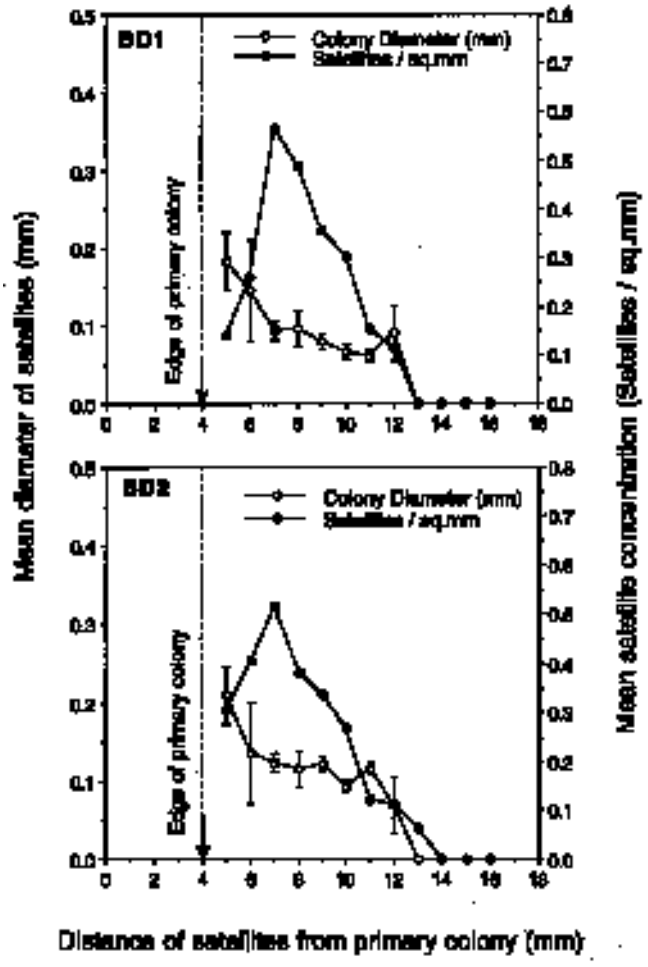

Fig. 2 Quantification of the interaction effects between primary and satellite colonies during community plating. Error bars are 95% confidence intervals based on the number of colonies present at each distance from the primary colony. Distances were measured from the center of the primary colony

Effect of the concentration of satellites on the strength of association The size and spatial distribution of satellites varied inversely with distance from the primary colony at the three dilutions tested (Fig. 5). The maximum increase in colony size (growth of satellites) as a function of distance was obtained at the highest dilution (when the lowest number of cells were inoculated; corresponding to an inoculated mean satellite concentration of 2.2×10^2 satellites/cm^2). The minimum increase was obtained at the lowest dilution (2.2×10^4 satellites/cm^2). In contrast, the maximum increase in observed mean satellite concentration (survival of the satellites) as a function of distance was obtained at the lowest dilution and the minimum increase was obtained for the highest dilution. At the lowest dilution,

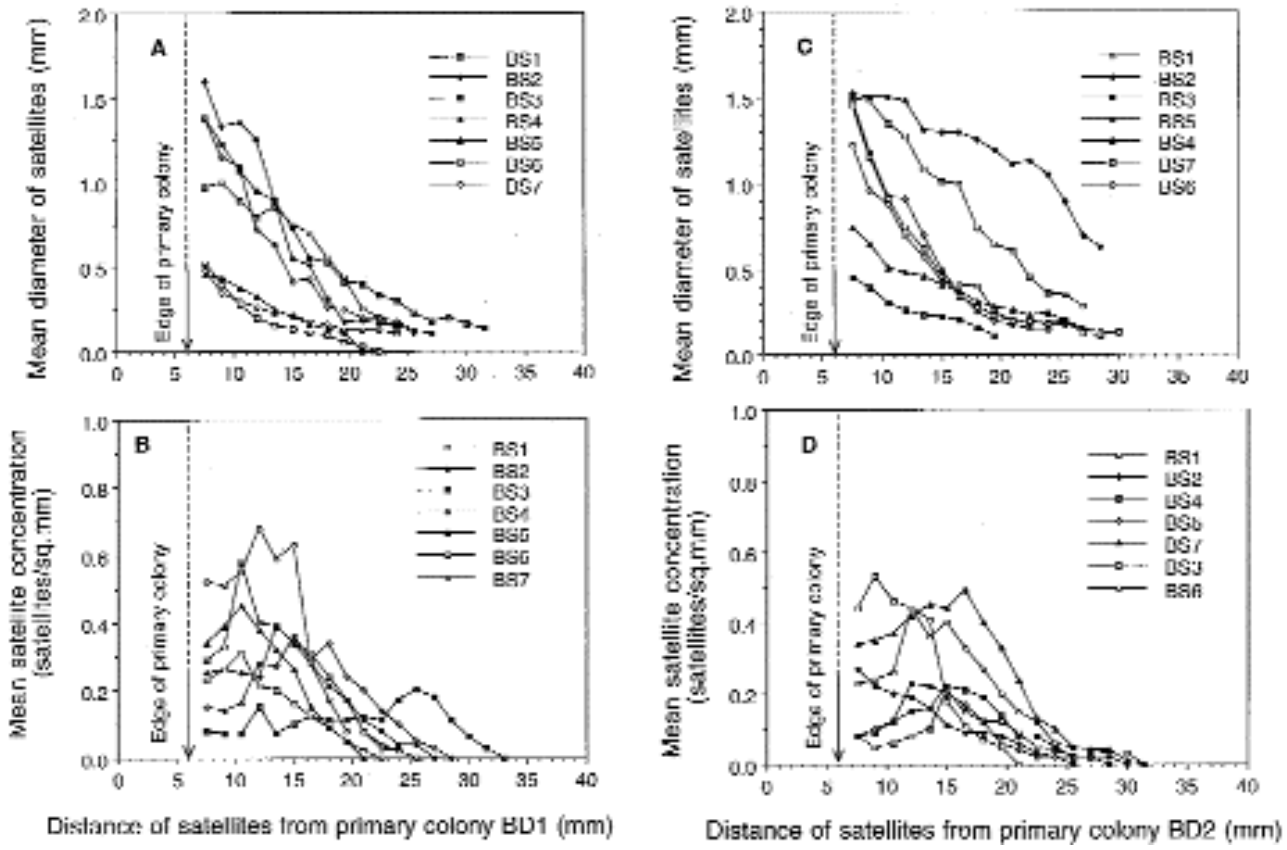

Fig. 4 Satellite analysis curves showing the size and spatial distribution of individual satellites as a function of distance from the primary colonies BD1 (A and B) and BD2 (C and D). Negative slopes of these curves indicate positive synergisms between individual satellites and the primary colony

(2.2×10^4 satellites/cm^2) the primary colony supported satellite development only to a distance of 25 mm (Fig. 5D). The distance from the primary colony to which satellite colonies could form, was extended at higher dilutions (2.2×10^2 and 2.2×10^3 satellites/cm^2) (Fig. 5E, 5F).

Influence of satellites on the primary colonies There was a significant reduction ($p < 0.01$) in the size of BD1 when cultivated in association with satellite colonies. Significant reductions in the size of BD2 were also noted, when associated with all satellite strains except BS4 and BS5.

Mechanism of satellite associations The effect of benzoate concentration on the development of associations between individual primary and satellite colonies was examined to determine if benzoate toxicity was the basis for the formation of such associations. The growth of primary colonies was inhibited at 1.0 and 1.5% benzoate concentrations. At lower concentrations (< 0.25% benzoate), satellite colonies could form, in association with the primary colony. However, all satellite morphotypes could develop independent of the primary colony only at much lower benzoate concentrations (0.015–0.05%). Even when (0.1%) glucose was added to the benzoate medium, satellite isolates were not able to grow independent of the primary colonies at higher benzoate concentrations (> 0.05%). All the satellite isolates were able to grow well, independent of the primary isolates, on minimal salts agar supplemented with glucose (no benzoate added). Neither agar plugs removed from areas adjacent to the spot-growth of primary isolates nor homogenates prepared from these plugs supported the growth of satellite strains on 0.15% BA.

Specificity of the BD1 and BD2 associations Cross-streak assays showed that both BD1 and BD2 were able to support the growth of organisms unrelated to the BD1 and BD2 associations on BA supplemented with glucose. These included a culture collection strain of *E. coli* (ATCC 25923) as well as MPT1 and MPT2, two other members of the TSB community from which the BD1 and BD2 associations were originally obtained.

Discussion

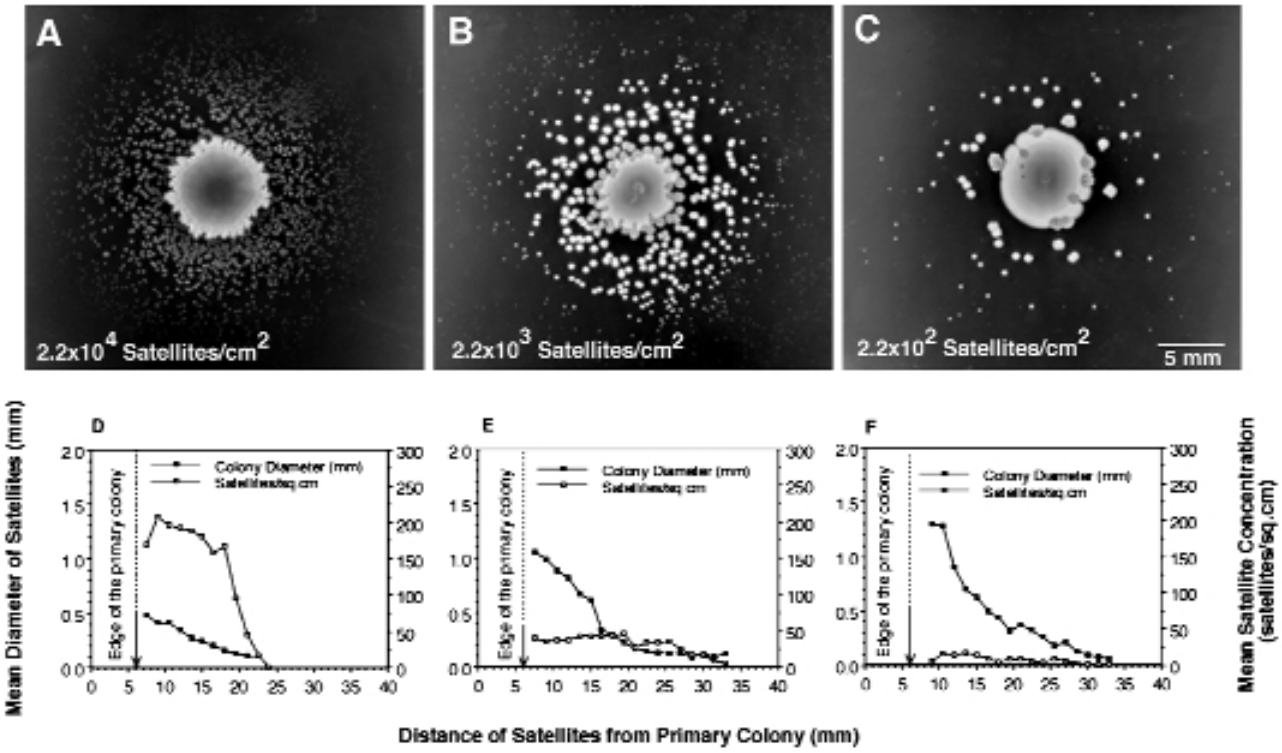

Fig. 5 Effect of satellite concentration (satellites/cm²) on the association between primary and satellite colonies was determined by plating various dilutions (A–C) of the log phase cells of BS2 on 0.15% benzoate agar (BA). BD2 was spot-inoculated at the center. Note that the positive effect of the primary colony tends to be masked by the self-inhibition of satellites when used at high satellite concentrations. Satellite analysis curves (D–F) show the size and spatial distribution of satellite morphotype BS2 as a function of distance from the primary colony BD2, at the three satellite concentrations indicated

The antimicrobial effectiveness of sodium benzoate has been widely recognized [12, 17, 20, 26]. However, the present study demonstrates the formation of protective synergisms involved in benzoate resistance and degradation leading to the reduced antimicrobial effectiveness of sodium benzoate. The protective synergisms were evident from (i) increased plating efficiencies (higher CFU/ml, determined based on plate counts) at the lower dilutions, when a TSB community was plated on 0.15% BA (Fig. 1) and (ii) the formation of satellite associations in which the primary colony enhanced the growth and survivability of the benzoate-sensitive satellite strains (Figs. 2 to 5).

Positive associations within benzoate-degrading biofilms that resulted in an enhanced plating efficiency when lower dilutions were plated on 0.15% BA have previously been reported [6]. It was suggested that the closer spatial positioning of the individuals at lower dilutions could facilitate associations and enhance the survival of the benzoate-sensitive members of the community. Density-dependent plating effects have also been reported with bacteria isolated from subsurface environments [Abstracts of the Conference on Multicellular and Interactive Behavior of Bacteria, ASM. MBL, Woods Hole, 1990, p. 20]. Thomas et al [22] showed the effect of spatial propinquity on the inhibition of subsurface colonies of *Listeria monocytogenes* by a nisin producing lactic acid bacterium. A separation distance of less than 100 µm from the lactic bacteria resulted in a 3.5 log reduction in the cell numbers of subsurface colonies of *L. monocytogenes*, as opposed to only a 0.9 log reduction in cell numbers at a greater separation distance of 3000 µm. As suggested previously [25], the propinquity or spatial separation between cells could influence the outcome of interactions among the bacteria in natural environments and model systems. Hence, an analysis of the effects of spatial separation should be an important consideration in screening for associative effects among bacteria as well as in the development of efficient methods for food preservation or biodegradation using degradative consortia and communities.

Positive associations among the members of the biofilm community also resulted in the formation of satellite colonies around larger, central colonies on 0.15% BA. However, the satellite effect was detected only when lower dilutions of the microbial community were plated on a high, inhibitory concentration of sodium benzoate. This is presumably due to the closer spatial positioning of bacteria at lower dilutions which facilitated interactions among the members of the microbial community. Although spot-inoculation and cross-streak assays

were useful to further confirm the positive interactions between individual primary and satellite isolates, they did not permit quantification of the relative effects of the association on the growth and survival of the organisms involved.

Quantitative analysis using the digital imaging procedure revealed that the size and number of satellite colonies decreased as a function of distance from the primary colony indicating the synergistic nature of the associations. This occurred when BD1 or BD2 associations which appeared on the plating-efficiency assay plates (Fig. 2) were analyzed as a whole or as binary pairs involving individual primary colony-satellite strains. During the analyses, the size of satellite colonies served as a measure of growth, and the number of satellite colonies detected per unit area served as a measure of the survival of benzoate-sensitive satellite isolates in association with a primary colony. When the interactions between individual primary and satellite isolates were analyzed, differences could be noted in the growth and survival of the same satellite as influenced by BD1 or BD2 (Fig. 5A–D) meaning that both primary isolates differed in their capacity to support the growth of various satellites.

Although, the growth stimulatory effect of the primary colonies upon the satellite isolates was evident from an analysis of size and spatial distribution of satellites around the primary colonies, the mechanism of the association was not clear. One of the requirements for understanding communities as causative agents of various biological processes is that the interactions among community members be understood [7]. Hence, the next set of experiments was aimed at determining the mechanism for the formation of satellite associations. Many documented satellite effects have been shown to be based on nutritional symbioses [2, 3, 14, 15, 19].

For example, two species of streptococci, *Streptococcus adjacens* and *S. defectivus* (responsible for human endocarditis) were characterized by their growth as small satellite colonies nutritionally supported by primary colonies [2]. Similar nutritionally variant streptococci (NVS) have previously been characterized as fastidious viridans group streptococci requiring supplemental cysteine or vitamin B6 (pyridoxal phosphate) [14] in complex media for growth, and form satellite colonies around several Gram-positive and Gram-negative bacterial strains [3]. Protection afforded by primary colonies against antibiotics has also been implicated in the formation of satellite effects [23]. Because of the rather refractory nature of the carbon substrate used (sodium benzoate), and because the degradation of benzoate by many pseudomonads has been well demonstrated [10], it is also possible that the satellites lacked the ability to degrade benzoic acid and depended on the primary colony for easily utilizable degradation intermediates. Thus, three mechanisms were hypothesized for the formation of the primary colony-satellite associations on BA. These included: (i) primary colonies supplied one or more nutritional factors that the satellite strains were unable to synthesize, (ii) satellite strains lacked the ability to degrade sodium benzoate and hence were dependent on the primary colonies for a supply of benzoate degradation intermediates, (iii) primary colonies protected the benzoate-sensitive satellites against inhibitory concentrations of benzoate by either reducing the concentration of sodium benzoate to non-inhibitory levels or by the production of specific or non-specific protective factors.

During the present study, the growth of satellites at high concentrations of benzoate (0.10–0.25%) was dependent on spatial propinquity with the primary colonies, even in the presence of an additional labile carbon source such as glucose. However, the satellite isolates were able to form colonies independent of the primary isolates at lower benzoate concentrations (0.015–0.05%). Satellite isolates were also able to grow on a minimal salts medium supplemented with glucose as sole carbon source (GA) in the absence of primary isolates. This suggested that the satellites were not dependent on a supply of growth factors by the primary colony. The ability of satellite isolates to grow independently of the primary colony at low concentrations of benzoate also suggested that satellite strains were able to utilize benzoate as sole carbon source, and the supply of the benzoate degradation intermediates by the primary colonies might not be the basis for the formation of the satellite associations. The growth of the satellite strains was inhibited at high benzoate concentrations even when provided with an additional, labile carbon source. Consequently, the most parsimonious explanation for the primary colony-satellite interactions is that the primary colonies played a protective role, allowing the development of satellite colonies at inhibitory concentrations of benzoate. The observation that the primary colonies were able to support satellite development to extended distances, when lower numbers of the satellite isolate was plated (at higher dilutions of satellites), suggested the possibility of a diffusible protective factor that diffused to extended distances at lower satellite concentrations.

However, neither the benzoate-agar plugs removed from areas adjacent to primary colonies, nor the homogenates prepared from these plugs did not support growth of satellite strains on 0.15% BA. This suggested that primary colonies resulted in a general toxicity reduction by benzoate degradation resulting in the satellite effect. The ability of the primary colonies to support the growth of unrelated organisms, as evidenced from cross-streak assays, indicates that the protection afforded by the primary colonies was not specific for satellites and confirms general toxicity reduction as a mechanism for the satellite effect. The fact that no synergistic effects could be detected due to interactions among multiple primary and satellite isolates also suggests that toxicity reduction was primarily responsible for the formation of satellite associations.

The possible sharing of the degradation intermediates between primary and satellite colonies is evident from the decreased mean satellite concentration immediately adjacent to the edge of the primary colony (Fig. 2, Figs. 4B and D) and

significant reduction of the size of primary colonies, upon adjacent inoculation with satellite isolates.

A number of factors such as (i) the levels and types of microorganisms in the system, (ii) presence or development of preservative-resistant strains of microorganisms (either due to their ability to degrade the preservative or other factors), (iii) formation and spatial distribution of synergistic bacterial consortia, (iv) reduced effective concentration of the preservative within the microenvironment of consortia, (v) use of an antimicrobial with a specific spectrum that might create favorable conditions for the proliferation of non-target organisms, (vi) other factors such as properties of the food material, its storage and influence of other preservation methods, etc. are known to affect the effectiveness of food-preservatives [5].

The results of the present study, however, suggest that the associative effects between preservative resistant strains and sensitive microbial strains (factors iii and iv) are more important. Testing the susceptibility of pure-cultured microorganisms to antimicrobials can sometimes give false-positive results. This occurs if the organism is susceptible to an antimicrobial agent when grown in pure culture, but not when grown in community culture if synergistic mechanisms provide resistance [4, 7]. Generally, the occurrence of detrimental bacteria in association with other bacteria may lead to the unforeseen persistence of unwanted organisms under inhibitory conditions. Similarly, identification of synergistic or protective interactions between the commensal microbiota and pathogens might be crucial in the choice and administration of methods to treat human illnesses. For example, Dykhuizen et al. [11] reported that failure of co-amoxyclav or penicillin in the treatment of Group A streptococcal (GAS) pharyngitis and its recurrence is associated with the presence of β-lactamase activity in commensal microbiota. Identification of synergistic interactions may also be useful in development of efficient systems for waste water treatment and bioremediation [25]. Thus the formation of protective synergisms as demonstrated in the present study, suggests that proliferation strategies based on association should be an important consideration, in order to fully understand and develop applications in the disciplines of food preservation, human health, and other environmental processes such as biodegradation.

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