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RESEARCH ARTICLE

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Adaptation of bacterial communities to environmental transitions from labile to refractory substrates

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Abstract The aim of this work was to assess the adaptation of bacterial communities to environmental transitions from labile to refractory substrates. This involved testing the hypothesis that bacteria self-organize and propagate not only as individual cellular systems, but also as functional sets of interacting organisms. A biofilm community was cultivated in a flow-cell irrigated with tryptic soy broth and subjected to a cyclic series of environmental transitions, from labile to refractory substrates, followed by a period of starvation (30 days). The appearance and disappearance of specific colony morphotypes when the emigrants were plated onto tryptic soy agar was used to monitor the restructuring of the community. Confocal laser microscopy of flow cells showed that these transitions decreased the biofilm thickness and coverage. Substrate shifts also changed the architecture of the biofilm communities. Repeated inoculation of flow-cell communities with a composite inoculum increased the number and diversity of emigrants. Their biofilms were thicker and covered a wider area than those of communities that had been inoculated only at the beginning of the experiment. With repeated inoculation, the time required for the community to restructure and stabilize decreased during most transitions. This suggested that organismal recombination acted as a mechanism of adaptation, enhancing the growth of microbial communities exposed to environmental stresses. Changes in the profiles of emigrants during the adaptation of biofilm communities to environmental transitions showed the appearance and disappearance of discrete sets of organisms. This suggested that the biofilm communities responded to environmental stresses as sets of interacting organisms. Enhanced growth of biofilm communities due to repeated environmental cycling suggested that the functionality of cellular positioning accrued from one cycle to the next and was thus heritable, although it was not necessarily genetically encoded.

Keywords Biofilms · Restructuring · Environmental transitions · Biofilm architecture · Microbial communities

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Introduction

The role of community-level bacterial strategies in biodegradation has been well established [22, 27]. In degradative microbial communities, the capability of breaking refractory organic compounds arises from physiological [20, 27] and genetic [12] interactions among community members. This capacity results from the metabolic diversity of its members, in terms of the number and variety of chemical bonds on which they can act [20]. Horizontal transfer of degradative plasmids among genetically unrelated bacterial cell lines [12] also enhances reproduction in degradative communities, because more degradative phenotypes appear. These adaptive strategies combine the adaptive potential of cells (spatial recombination of molecules in cells) with the adaptive potential of communities (spatial recombination of organisms in the communities). Thus, when these communities adapt to environmental perturbations, they undergo restructuring on multiple spatial scales [18, 23].

Synergisms among the members of communities are required for the optimal proliferation of the communities themselves [5, 6, 7, 8], just as cellular and molecular synergisms have been optimized for the proliferation of cells and molecules. In fact, the restructuring of communities not only involves gene mutations and

recombination, but also recombination of cells and organisms both within a single community and among communities. However, the role of external organismal recombination (i.e. the effects of immigration and emigration) in the process of adaptation of bacterial communities to environmental stresses has not been studied from this perspective. If adaptation to some kind of stress occurs at multiple spatial levels of organization, including the community, then an environmental shift should elicit co-ordinated responses among the community members. Thus, the changes in community structure profile in response to substrate transitions should show sets of inflections, as opposed to members responding individually.

This study tested the existence of community adaptation strategies. We subjected a microbial community to cyclic environmental stresses, from labile to refractory substrates [from triptic soy broth (TSB) to benzoate, then to 2,4,6-trichlorobenzoic acid (2,4,6-T)] and to starvation. Then, we examined the role of multiscalar recombination in the adaptation of biofilm communities at various stages of environmental transition.

Materials and methods

Bacterial communities and culture conditions

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 (M-salts) solution [27]. This was used to inoculate a flow cell [21] (channel dimensions: 1×3×40 mm) continuously irrigated with 0.3% (w/v) TSB broth at a laminar flow velocity of 0.1 mm/s. A quasi-steady state biofilm community developing on the solid–liquid interface was determined from the stability of its thickness, area coverage and overall architecture, as well as from the number and diversity of emigrants. The quasi-steady state community was then subjected to a sudden environmental transition (to sodium benzoate as sole carbon source). Subsequent transitions consisted of a shift to 2,4,6-T as sole carbon substrate and then to a state of starvation (no carbon). The substrate shifts were accomplished by changing the flow-cell irrigation medium from 0.3% (w/v) TSB to M-salts supplemented with 0.15% (w/v) sodium benzoate, 0.05% (w/v) 2,4,6-T, or no carbon (when subjected to starvation), depending on the stage of the cycle. All chemicals were procured from Sigma Chemical Co., St. Louis, Mo.

In the first cycle (Cycle I-S), the communities were subjected to sudden environmental transitions that consisted of instantaneous shifts in the cultivation medium. The second set of cycles involved either sudden or gradual (stepwise) environmental transitions (Cycle II-S and Cycle II-G respectively; Fig. 1). During gradual transitions, each shift extended over a period of 30 days and comprised 10 steps (of 3 days each). Each step consisted of a 0.1-fold decrease in the concentration of the first substrate, accompanied by a 0.1-fold supply of the second substrate. After 30 days, the concentration of the first substrate in the irrigation medium was zero and that of the second was at its maximum.

During both cycles, biofilm communities that were repeatedly inoculated were compared with the communities inoculated only at the beginning of the experiment. Repeated inoculation consisted of inoculation at 12-h intervals, using 0.5 ml of a freshly prepared composite inoculum.

Monitoring biofilm reorganization

During Cycle I-S and Cycle II-S (Fig. 1), the thickness, percent area coverage and architecture of biofilm communities were

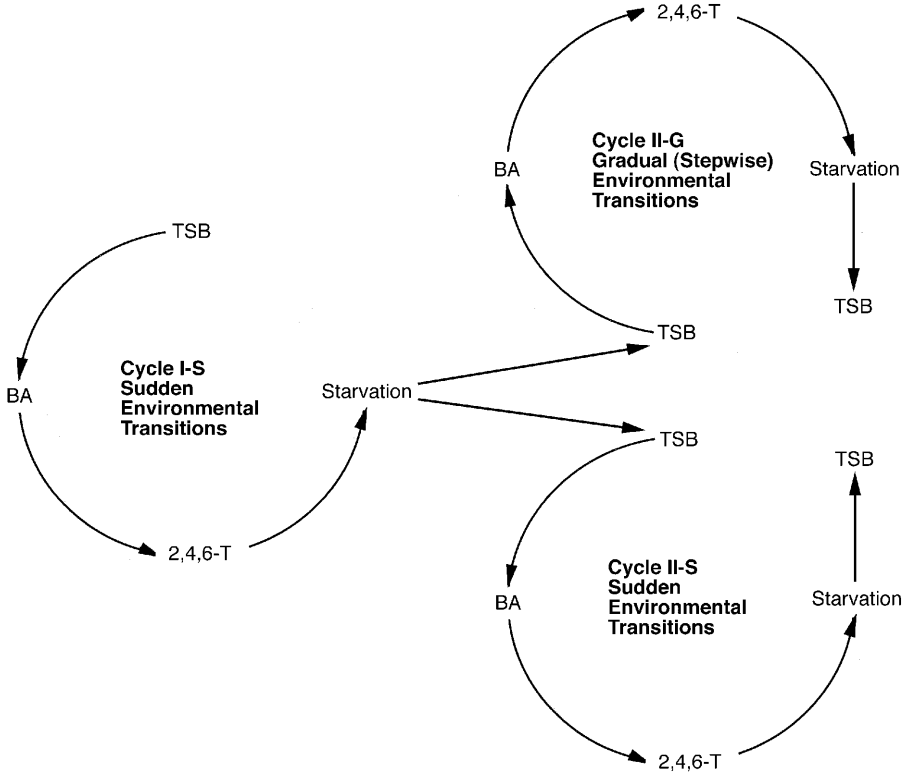

Fig. 1 A flow-cell community cultivated on tryptic soy broth (*TSB*) was subjected to three environmental cycles as described in the text, *BA* Benzoate, *2,4,6-T* 2,4,6-trichlorobenzoic acid

measured using scanning confocal laser microscopy (SCLM). In Cycle I-S, the measurements were done at 12-h intervals, until the community reached a quasi-steady state, and at longer intervals on the stable biofilm community. In Cycle II-S, SCLM measurements were done less frequently (once before the transition and twice after the community stabilized in the new environment). Biofilms negatively stained with 0.1% w/v fluorescein (Sigma Chemical Co., St. Louis, Mo.) were scanned and digitized using a Bio-Rad MRC-600 Lasersharp fluorescence scanning confocal laser system (Bio-Rad Microscience, Canada), mounted on a Nikon F×A microscope, under a 60×, 1.4 NA oil immersion objective (Nikon Corporation, Tokyo). An argon laser operated at 25 mW power and 1% beam transmission (maximum excitation at 488 nm and 514 nm) was used in the excitation of the fluorophore. The images were collected using a Kalman filter. A Northgate 80846 host computer was used to display the images. The focusing and stage control were computerized. The images were saved on an optical disc using a Corel-940 optical drive (WORM). The primary images were analyzed using Bio-Rad software in the Northgate host computer. The percent biofilm area and the biofilm thickness at each observation period were respectively the averages of 10 and 20 replicate measurements. Communities subjected to gradual transitions (Cycle II-G) were not examined by SCLM.

Monitoring the diversity of emigrant populations

The diversity of the emigrants (as measured by richness and Shannon indices) was monitored at 12-h intervals until the communities reached a quasi-steady state and, less frequently, in stable biofilm communities. These determinations were done prior to the laser microscopy of biofilm architecture in Cycle I-S and Cycle II-S. In Cycle II-G, the emigrants were enumerated just before each stepwise transition. For this purpose, the flow-cell effluent was collected for 1 h and plated onto 0.3% (w/v) TSB agar in triplicate. The plates were incubated at 22 °C for 7 days and the number of various colony morphotypes that appeared on countable plates of highest dilution, usually containing 30–100 colonies, were counted. Colony morphotypes were determined, based on the descriptions of Benson [4] and Eklund and Lankford [11]. Richness was based on the number of distinct colony morphotypes observed. The Shannon index of diversity $H = -\Sigma(p_i \times \log 2 p_i)$ was then calculated. The factor p_i is the number of isolates classified as a certain morphotype divided by the total number of isolates in the sample being analyzed.

Results

Biofilm thickness and percent area coverage

Shifts in carbon substrates, from TSB to benzoate to 2,4,6-T, resulted in a general reduction in the thickness and percent area coverage of biofilm communities. In Cycle I-S, when cultivated using TSB, the thickness and percent area coverage of repeatedly inoculated and initially inoculated biofilm communities did not differ significantly ($P < 0.05$). When subjected to a substrate shift to sodium benzoate, the thickness and area coverage of both kinds of communities decreased significantly. However, inoculated biofilm communities repeatedly had a significantly ($P < 0.01$) greater thickness (13.1 μm) and percent biofilm area coverage (14.3%) than those inoculated only initially (10.1 μm and 8.1%, respectively). During subsequent substrate shifts, repeated inoculation resulted in thicker biofilms, which had wider coverage than those of initially inoculated communities. There were further decreases in biofilm thickness and coverage upon subsequent substrate shifts, although they hardly changed between transitions. At the end of Cycle I-S, an environmental shift from a state of starvation (no carbon) to growth on a labile carbon (TSB) resulted in significant increases ($P < 0.01$) in the thickness and percent area coverage of both kinds of communities. In contrast to the TSB communities of Cycle I-S, the TSB repeatedly inoculated communities of Cycle II-S were thicker and covered a wider area (20.9 μm and 26.34%, respectively) than those initially inoculated (18.5 μm and 23.87%, respectively). The overall trends in biofilm thickness and coverage during Cycle II-S were similar to those of Cycle I-S, but biofilms were more robust during Cycle II-S.

Biofilm architecture

Substrate shifts also changed the architecture of the biofilm communities (Fig. 2). Labile carbon substrates such as TSB resulted in randomly distributed cells, whereas cultivation using a more refractory, ring-structured compound, such as sodium benzoate, resulted in the formation of more organized cell clusters. Cultivation using 2,4,6-T produced distinct tree-like arrangements of cells in biofilm communities. Similar architectural features occurred in both repeatedly inoculated and initially inoculated biofilm communities grown on the same substrate. The changes in biofilm architecture followed the same trend (from less organized to more organized) during Cycle II-S.

Emigrant cell numbers

Irrespective of the frequency of inoculation, the number of emigrants decreased in response to substrate refractoriness in both Cycle I-S and Cycle II-S (Fig. 3). Starvation (no carbon) resulted in the lowest cfu/ml values. Cycle II-G showed similar trends in the number of emigrants. The numbers of emigrants during Cycle II-S were generally higher than in Cycle I-S. The numbers of emigrants (cfu/ml) were in all cycles significantly higher ($P < 0.01$) in the repeatedly inoculated communities than in those inoculated initially.

Structure and diversity of emigrant populations

Substrate shifts during both kinds of cycles resulted in the reshuffling of members in biofilm communities, as revealed by the changes in the numbers of colony morphotypes detected when we plated flow-cell emigrants. Figure 4 shows the process of restructuring in repeatedly and initially inoculated communities of Cycle I-S, in response to an environmental transition from sodium benzoate to 2,4,6-T as sole carbon source. When the initially inoculated community was subjected to the

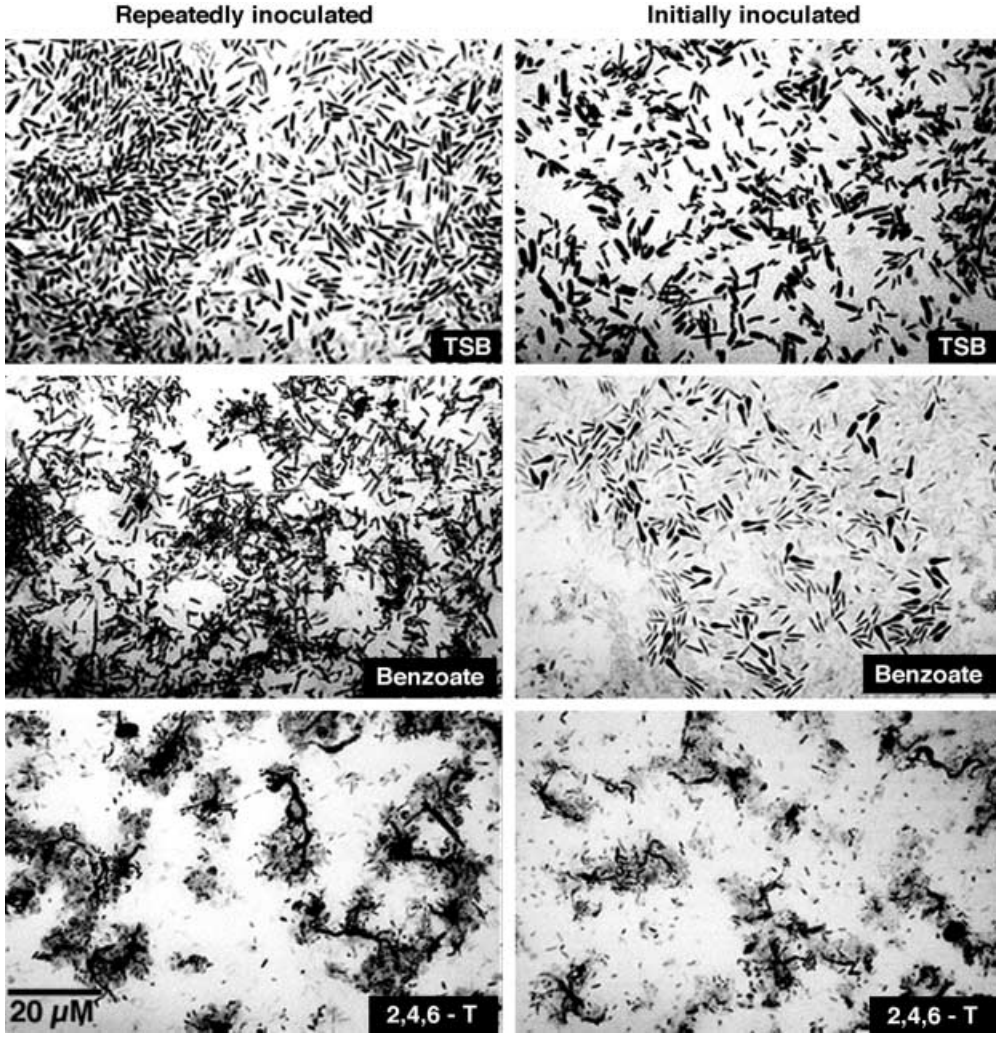

Fig. 2 Confocal scanning laser micrographs of repeatedly inoculated and initially inoculated biofilm communities subjected to sudden environmental transitions from TSB to benzoate to 2,4,6-T during Cycle I-S

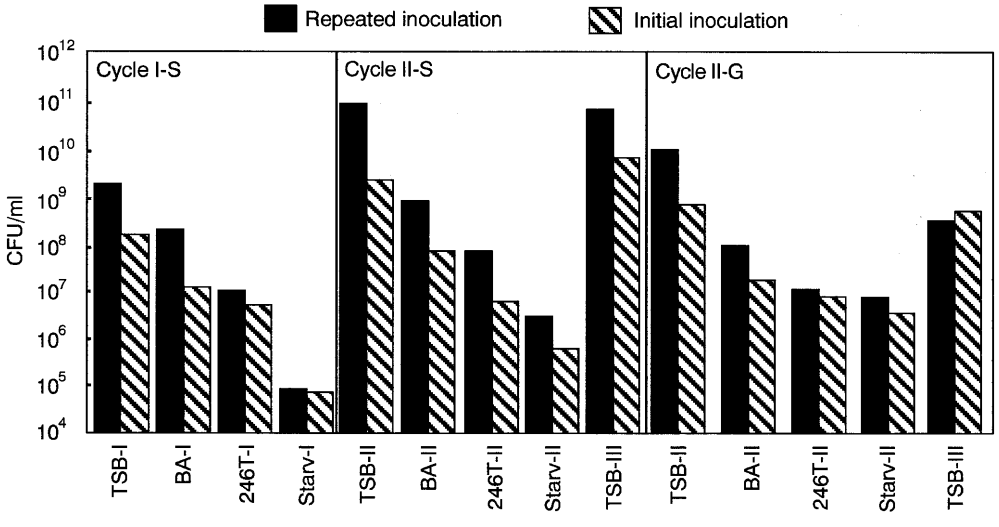

Fig. 3 Relationship between the refractory nature of the substrate and cell counts of emigrant biofilm communities. *Starv* No carbon source, *246T* 2,4,6-trichlorobenzoic acid, *TSB* tryptic soy broth, *BA* benzoic acid

substrate shift, the patterns of restructuring of emigrant populations showed that one morphotype (mpt-6) disappeared, whereas four new morphotypes (mpt-11, 14, 15, 16) appeared in response to the transition. More drastic changes occurred in the repeatedly inoculated biofilm community, which lost three of its morphotypes and gained seven new members. The appearance, propagation and disappearance of some of the emigrants occurred as sets. For example, morphotypes 7 and 8 were present throughout the transition and

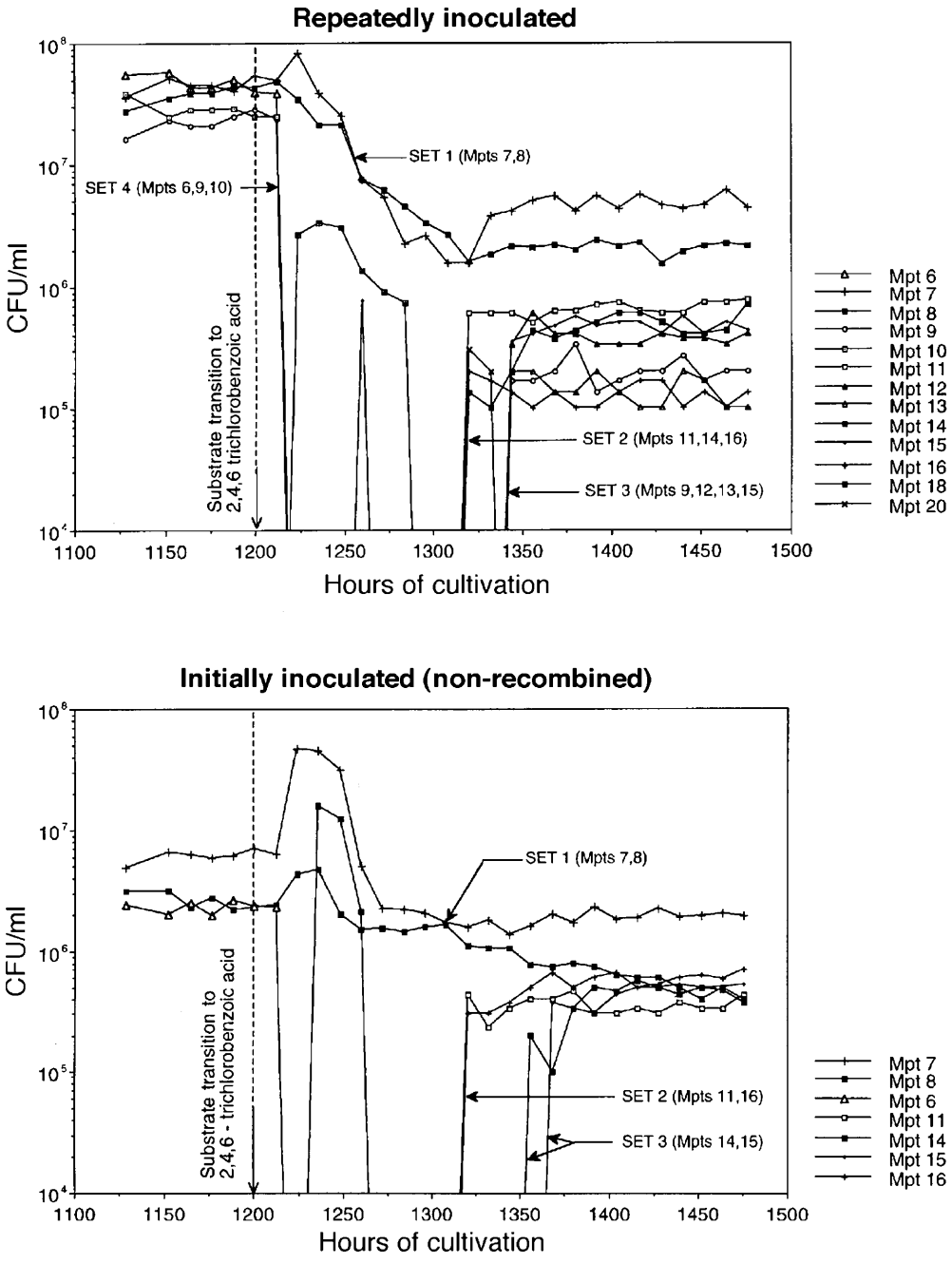

Fig. 4 Effect of community restructuring on the emigration patterns detected when repeatedly inoculated and initially inoculated biofilm communities were subjected to a substrate transition from benzoate to 2,4,6-T. *Mpt* Morphotype

seemed to propagate as a set (Set 1). This set was present in both kinds of communities. When those repeatedly inoculated were subjected to the substrate shift, morphotypes 11, 14, and 16 and morphotypes 9, 12, 13 and 15 appeared as two distinct sets (Set 2 and 3, respectively), whereas morphotypes 6, 9 and 10 disappeared as a set (Set 4). Similarly, during adaptation of the initially inoculated biofilm community, two distinct sets [morphotypes 11, 16 (Set 2) and morphotypes 14, 15 (Set 3)] appeared. When communities were subjected to stepwise environmental transitions, some morphotypes appeared, disappeared or propagated as sets. During most transitions, repeatedly inoculated communities adapted to substrate shifts more rapidly than those inoculated only initially. For example, when subjected to a substrate shift from benzoate to 2,4,6-T, repeatedly inoculated biofilm communities required a shorter period (140 h) for restructuring and stabilization than initially inoculated communities (175 h) (Fig. 4).

Recoverable diversity of the emigrants measured from colony morphotypes was generally higher in repeatedly inoculated communities of both Cycle I-S and Cycle II-S than in the initially inoculated communities grown on the same substrate. However, Shannon diversity indices did not show any specific trend based on the refractory nature of the substrate, the nature of the transition (sudden or gradual) or the cycle of adaptation (I or II; data not shown).

Discussion

Earlier studies on the effects of environmental perturbations on microbial communities focused mainly on the impacts of organic carbon amendments by pulse-feed, step-feed or periodic-feed patterns [16, 18, 23]. These substrate perturbations led to changes in the structure of microbial communities as a mechanism of adaptation. In nature, environmental perturbations are much more complex and comprise physicochemical (changes in the environmental concentrations and gradients of growth substrates, pH, redox potential, etc.) and biotic (effects of micro- and macrobiota sharing the same ecosystem) factors. This is especially obvious in aquatic ecosystems, where flow influences both the flux of nutrients and an influx and efflux of other microorganisms. In such systems, planktonic bacteria can be considered dynamic "gene pools" that can colonize new environmental niches. Their significance in the new environment might be reflected by shifts in the structure and metabolic capability of the native community. If proliferation occurs at the level of communities as well as at the level of individuals, then repeated inoculation with a diverse inoculum should optimize both community-level adaptation in terms of the time required for the community to restructure and stabilize and the diversity and productivity of the resulting assemblage of organisms. Hence, we assessed the effect of repeated inoculation as an external source of organismal recombination (a process analogous to genetic recombination at the cellular level), under conditions of cyclic environmental shifts.

The results showed a decline in the thickness and area coverage of biofilm communities and a decline in the numbers of emigrants upon successive substrate shifts. Repeated inoculation of biofilm communities enhanced adaptation. This suggested that communities need synergisms (resulting from both internal and external recombination processes) for their optimal growth, just as individuals need molecular and cellular synergisms for their optimal growth. James et al. [19] indicated that interspecies interactions influence the structure and physiology of biofilm communities during the course of their formation. Repeated inoculation may have allowed a larger genetic pool for those interactions. This is consistent with the proliferation theory [5, 6, 7, 8], which suggests that bacterial self-organization not only involves mutation and recombination of genes, but also the restructuring of communities (immigration, emigration, reshuffling of members within a community) and the spatial repositioning of organisms within geographic boundaries (the flow cell and its surface environments).

Techniques for the qualitative analysis of microbial community structure include ribosomal RNA (rRNA) analysis using taxon-specific probes [13], rRNA sequence comparisons [10], whole community DNA hybridization [15], whole community phospholipid fatty-acid profiles [26] and carbon source utilization profile analysis using Biolog microtiter plates [14]. Only some of these methods can account for the overall changes in community structure; and others are limited in their application when the actual process of restructuring has to be described. In this work, we used colony morphotypes that appeared when the effluent obtained from flow cells was plated on TSB agar to quantify emigration (the number and diversity of emigrant populations) from the community. Haldeman and Amy [17], in their studies on the colony morphotypes of bacterial isolates obtained from various rock samples, concluded that colony morphotypes can be considered as biotypes and could provide an accurate basis to define recoverable diversity. Colony development had been previously used to characterize bacterial communities on wheat roots [9]. The selectivity of plating media and culture methods was unavoidable in this process. We followed specific morphotypes through each transition, using countable plates of the lowest dilution containing 30–100 colonies, which we incubated for the same duration. So, colony sizes and morphologies were comparable between two sampling periods.

Atlas [2] suggested that diversity measurements would be useful to monitor changes due to ecosystem perturbations and that the degree of diversity is lower in communities under stress conditions. In the case of co-metabolic interactions and sequential degradation of pesticides, species diversity is crucial to enable the breakdown of complex bond structures. This seems to imply that stressed communities will be diverse. During the present study, the diversity changes in response to substrate stress did not follow a specific pattern. However, emigrants of repeatedly inoculated biofilm communities tended to be richer and more diverse than emigrants of initially inoculated communities. This might be due to the immigration of new members into the community.

We used repeated environmental cycling to know whether pre-exposure of a microbial community to a refractory organic compound would enhance the community's ability to utilize the same compound upon subsequent exposures. Adaptation of microbial populations to use either refractory or toxic compounds as carbon and energy sources has been well demonstrated [1, 3, 24]. Adaptation of microorganisms to high toxicant concentrations would play a major role in biodegradation [25]. Spain and van Veld [24] defined adaptation in the microbial community as any change that increases the rate of transformation of a test compound as a result of a previous exposure to that compound. The higher cfu/ml of emigrants that we observed over Cycle II-S and Cycle II-G might have resulted from pre-exposure of the communities to these substrates during Cycle I-S. Enhanced growth of biofilm communities due to repeated environmental cycling suggests that the cellular positioning information accrued from one cycle to the next and may have been heritable.

A closer analysis of the emigration patterns that resulted from sudden environmental transition (Fig. 4)

indicated that substrate perturbations led to the collapse of existing community structures. However, the continuous process of recombination probably led to the development of new, stable sets of emigrant populations. The emigration patterns of all communities showed the appearance or disappearance of sets of organisms during bacterial adaptation to refractory substrates. During a sudden environmental transition from benzoate to 2,4,6-T, the appearance (Sets 2, 3), disappearance (Set 4) and propagation (Set 1) of stable sets of organisms suggests that the organisms in these sets might be related and that bacterial adaptation to environmental transitions may occur as functional sets of interacting organisms.

Communities were subjected to gradual environmental transitions to better understand the process of restructuring in response to environmental transitions. Due to difficulties in developing smooth temporal concentration gradients of two substrates, we applied stepwise concentration gradients. Although stepwise transitions were not useful to define the community boundaries or to detect the presence of ecotones (where one set of community-level relationships disappear and new set of relationships appear), they were just as useful as sudden environmental transitions to detect groups of organisms that appeared or disappeared as sets. This again suggested that the biofilm communities adapted to environmental stresses as sets of interacting organisms, instead of functioning exclusively as unrelated individuals.

Each community is a network of organisms that have adapted to propagate both as individuals and as part of one or more associations. Networking of organisms in a community might allow efficient use of environmental resources and production of favorable microenvironments within unfavorable macroenvironments [5, 6, 7, 8]. Bacteria may respond to environmental stresses as sets of interacting organisms, as revealed by the appearance and disappearance of sets of organisms during adaptation of flow-cell communities to substrate shifts. Continual inoculation as a mechanism of recombination led to more rapid adaptation and enhanced growth of microbial communities subjected to environmental transitions, which suggested that proliferation might result from recombination events at multiple levels of biological organization. Enhanced growth of biofilm communities when they were subjected to a second set of sudden environmental transitions suggests that the cellular positioning information accrued from one cycle to the next may have been heritable.

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