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Effects of community composition and growth rate on aquifer biofilm bacteria and their susceptibility to betadine disinfection

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Summary

Biofilm formation and function was studied in mixed culture using 20 bacterial strains isolated from a karst aquifer. When co-cultured in a glucose-limited chemostat, *Vogesella indigofera* and *Pseudomonas putida* were the dominant planktonic and biofilm organisms respectively. Biofilm formation and resistance to the iodine disinfectant betadine were then studied with monoculture and binary cultures of *V. indigofera* and *P. putida* and a 20-strain community. Biofilm population size [measured as colony-forming units (CFU) cm^{-2}] increased with increasing species diversity. Significantly larger populations formed at dilution rates (DRs) of 0.0083 h^{-1} than at 0.033 h^{-1}. *P. putida* populations were higher and *V. indigofera* lower in binary than in monoculture biofilms, suggesting that *P. putida* outcompeted *V. indigofera*. In binary biofilms, *V. indigofera*, a betadine-resistant organism, enhanced the survival of *P. putida*, a betadine-susceptible organism. In the 20-strain biofilms, this protective effect was not observed because of low concentrations of *V. indigofera* ($<$ 1% of the total population), suggesting that resistant organisms contribute to overall biofilm disinfectant resistance. Growth at 0.033 h^{-1} enhanced survival of *V. indigofera* biofilms against betadine. Although DR did influence survival of the other communities, its effects were neither consistent nor significant. All told, biofilm formation and betadine resistance are complex phenomena, influenced by community composition, growth rate and betadine concentration.

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Introduction

Many studies have focused on adhesion and biofilm formation by planktonic monocultures, e.g. *Pseudomonas aeruginosa* (reviewed in Costerton *et al*., 1995). Although prominent in nature, much less information is known about mixed population biofilms. Bacteria do not have uniform colonization and physiological properties (Fletcher, 1991), a feature that enables them to utilize different ecological niches. Therefore, one would predict that increasing species diversity of planktonic bacterial communities would lead to increased species diversity and overall cell density within biofilms. Similarly, one would also anticipate that growth rate would affect species composition and biofilm formation. Chemostat studies (primarily of monocultures) have shown that both growth rate and the properties of individual species do influence the formation of biofilms and the subsequent physiology of bacteria within biofilms (Pedersen *et al*., 1986; Anwar *et al*., 1990). In contrast to planktonic bacteria, biofilm bacteria are typically quite resistant to disinfectants (Cargill *et al*., 1992). One disinfectant commonly used in the biomedical field is povidone iodine (betadine) (Fleischer and Reimer, 1997). Biofilm resistance to betadine has been documented with batch-grown monoculture biofilms of *P. aeruginosa* (Brown *et al*., 1995), but has not been investigated under varying growth rates nor with mixed population biofilms. As naturally occurring bacterial biofilms rarely occur as monocultures, it is important to establish the effect of growth rate and species interactions on biofilm formation and to establish the population dynamics and susceptibility of component species to antimicrobial agents in mixed culture systems. In this study, we investigated the roles of community composition and growth rate on the propensity of chemostat-grown cultures to form biofilms, the relative abundance of species within binary biofilms and the subsequent susceptibility of biofilm bacteria to betadine. We examined bacteria isolated from the Edwards Aquifer, an environmentally sensitive habitat of several endemic organisms and the major source of groundwater in south central Texas, USA (Longley, 1994). The bacterial communities used included monocultures of

Table 1. Twenty bacteria were isolated from the Edwards Aquifer (a karst aquifer located in South Central Texas USA) and utilized for the formation of mixed culture biofilms.

Organism	Number of Isolates
Vogesella indigofera[a]	1
Pseudomonas putida[a]	1
Micrococcus luteus	1
Micrococcus nishinomiyaensis	1
Staphylococcus xylosus	1
Aeromonas hydrophila	1
Pseudomonas sp.*	4
Aeromonas sp.	1
Acinetobacter sp.	2
Alcaligenes sp.	2
Bacillus sp.*	3
Chromobacterium sp.*	1
Flavobacterium sp.	1

Each isolate was identified on the basis of published characteristics using biochemical and morphological attributes (Amy *et al*., 1992). Organisms which had been previously described in the Edward's Aquifer are designated by an asterisk (Koehn *et al*., 1991).
a. Identity confirmed using 16S rRNA sequencing (Reeves *et al*., 1995).

Pseudomonas putida and *Vogesella indigofera*, a binary culture of *P. putida* and *V. indigofera* and a community of 20 isolates that included *P. putida* and *V. indigofera*.

Results

Aquifer isolates

Originally, 32 isolates were obtained by enrichment culturing and growth on R2A agar (Difco). From these 32 isolates, 20 could grow on a defined minimal salts medium supplemented with glucose (described below). The identifications of these 20 isolates, as 13 species, are listed in Table 1. Although some of these organisms had been previously described in the Edwards Aquifer (Koehn *et al*., 1991), most of these strains represented new isolates. Of interest, very little work has been reported on two of these isolates, *V. indigofera* (Grimes *et al*., 1997) and *Micrococcus nishinomiyaensis* (Kocur *et al*., 1975). As revealed by transmission electron microscopy (Tolson *et al*., 1995), only *P. putida* had extensive piliation, which may partially explain its dominance in mixed culture biofilm populations. The identities of *P. putida* and *V. indigofera* were confirmed by partial sequencing of the 16S rRNA fraction of each organism.

Effects of community composition and dilution rate on total biofilm numbers

After 6 days, the colony-forming units (CFUs) of all planktonic chemostat cultures stabilized at 1.7–3.4 $\times$ 10^8 CFU ml^{-1}. Monoculture biofilm numbers of *P. putida* and *V. indigofera* did not differ within either dilution rate (DR) (Table 2). Monocultures of *V. indigofera*, binary and 20-strain populations all produced biofilms with higher CFU counts when grown at a DR of 0.0083 h^{-1} than at a DR of 0.033 h^{-1} (Fig. 1, Table 2). Total bacterial numbers as indicated by CFUs in 20-strain biofilms were significantly greater than all other cultures within both DRs (Fig. 1). The CFUs of binary biofilms was higher than *P. putida* monoculture biofilms at both DRs and was higher than *V. indigofera* monocultures at DR 0.0083 h^{-1}. This set of results suggests differential community responses to DR.

Table 2. Effect of community composition and dilution rate on biofilm numbers[a] as determined by two-way ANOVA.

Dilution rate effects	
Community composition	Comparison of dilution rates
Pseudomonas putida	0.0083 h^{-1} = 0.033 h^{-1} NS[b]
Vogesella indigofera	0.0083 h^{-1} > 0.033 h^{-1}***
Binary	0.0083 h^{-1} > 0.033 h^{-1}**
Twenty strain	0.0083 h^{-1} > 0.033 h^{-1}***
Community comparison	
DR = 0.0083 h^{-1}	DR = 0.033 h^{-1}
P. putida = *V. indigofera* NS	*P. putida* = *V. indigofera* NS
P. putida = Binary**	*P. putida* = Binary*
P. putida < 20 strain***	*P. putida* < 20 strain***
V. indigofera = Binary NS	*V. indigofera* < Binary***
V. indigofera < 20 strain***	*V. indigofera* < 20 strain***
Binary < 20 strain***	Binary < 20 strain***

a. Calculated as $\log_{10}$ (CFU cm^{-2}).
b. Significance denoted by *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, no significant difference at $P = 0.05$.

The effect of betadine disinfection

Initial experiments (data not shown) showed that a 3 min

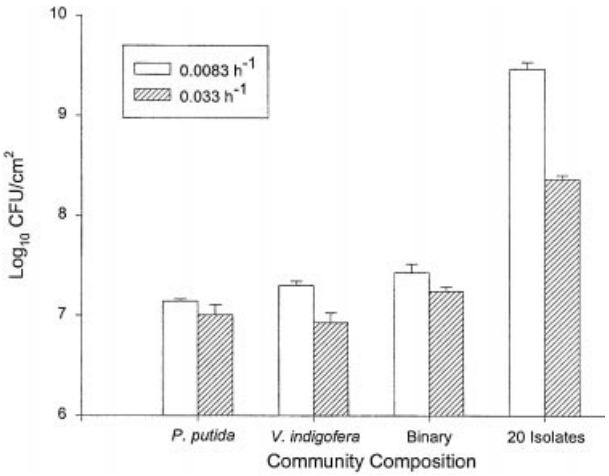

Fig. 1. $\log_{10}$ CFU cm^{-2} bacteria attached to silicon discs for the four bacterial communities at DR of 0.0083 h^{-1} and 0.033 h^{-1}. Error bars in this and all figures represent standard errors of the mean and are too small to be seen in some instances.

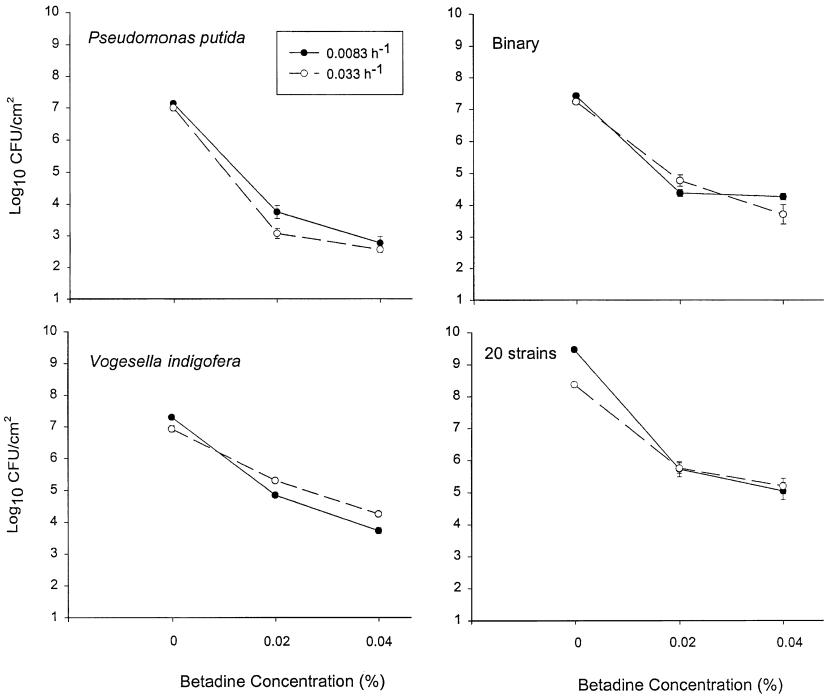

Fig. 2. Log_{10} CFU cm^{-2} of the four biofilm communities at DRs of 0.0083 h^{-1} and 0.033 h^{-1} at 0.02% betadine and 0.04% betadine.

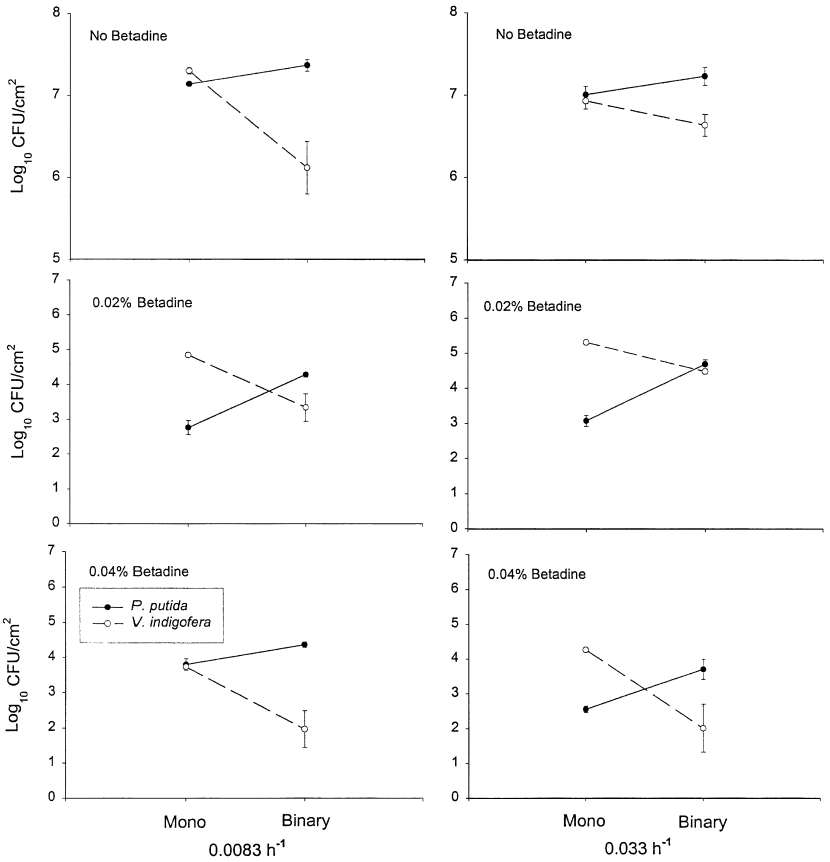

Fig. 3. Log_{10} CFU cm^{-2} of *P. putida* and *V. indigofera* present in mono- and binary culture biofilms at DRs of 0.0083 h^{-1} and 0.033 h^{-1}.

exposure to a 0.02% (v/v) dilution of betadine reduced culturable planktonics from 10^8 to $< 10^2$ CFU ml^{-1} for the most resistant community (*V. indigofera*) and from 10^8 to $< 10^1$ for the other three communities. Total biofilm cell concentrations at different DRs and betadine concentrations are shown in Fig. 2. The major factor influencing survival against 0.02% betadine appeared to be the presence of *V. indigofera* in the biofilm. In binary communities (Fig. 3), *V. indigofera* represented approximately 10–50% of the population at 0.02% betadine and 1% of the population at 0.04% betadine concentrations. We estimated the *V. indigofera* to be less than 1% of the total bacterial population in the 20-isolate biofilm community. Survival (Fig. 4) was highest in *V. indigofera* monoculture biofilms, intermediate in binary culture biofilms and lowest in the 20-strain and *P. putida* monoculture biofilms. In the 20-strain biofilms, we estimated the concentration of *V. indigofera* to be $< 1\%$ of culturable bacteria. The concentration of *V. indigofera* and *P. putida* in binary culture biofilms (addressed below) is shown in Fig. 3. DR did not have a consistent effect on survival to betadine disinfection (Fig. 2, Table 3). As shown in Fig. 4, survival of *P. putida* increased significantly in binary culture compared with monoculture under three of four conditions tested (Table 3). In contrast, the only significant difference in survival observed for *V. indigofera* was a significant decrease at a betadine concentration of 0.04% compared with 0.02% at the high DR. Overall, biofilm survival in all populations was less at 0.04% betadine than at 0.02% betadine owing to the strength of the disinfectant.

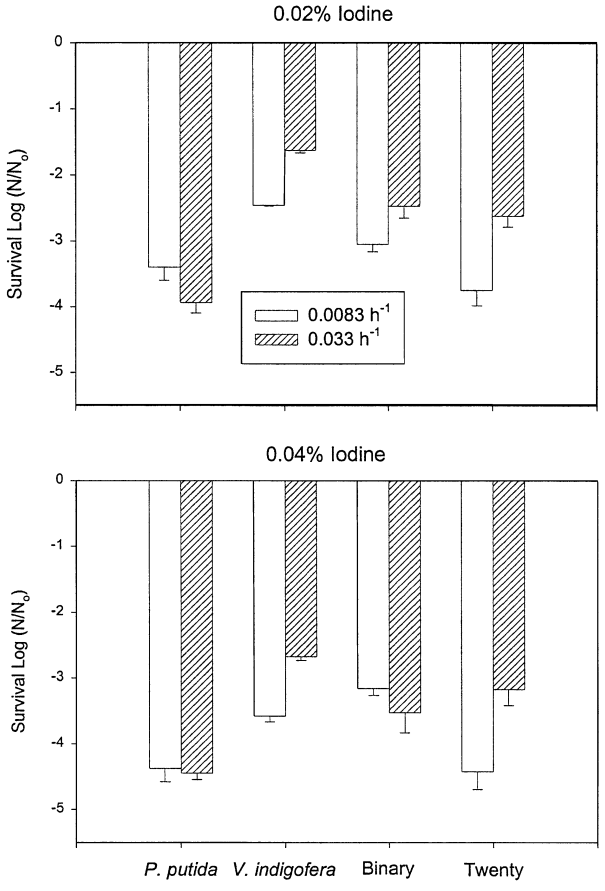

Fig. 4. Survival of *V. indigofera* and *P. putida* in mono- and binary culture biofilms after exposure to 0.04% or 0.02% dilution of betadine. Survival calculations are described in the text.

Interactions of V. indigofera *and* P. putida *in binary biofilms*

Mean planktonic binary population composition at DR 0.0083 h^{-1} of *P. putida* and *V. indigofera* in the chemostat culture was 52% and 48% respectively. At DR 0.033 h^{-1}, however, the percentage of planktonic *P. putida* and *V. indigofera* in the chemostat culture shifted to 79% and 21%, respectively, indicating that *P. putida* could outgrow *V. indigofera* at higher glucose concentrations. In the absence of betadine, the numbers of *V. indigofera* in binary biofilms decreased, and the numbers of *P. putida* increased significantly compared with monoculture biofilms at DR 0.0083 h^{-1} but not at DR 0.033 h^{-1} (Table 4, Fig. 3). In the presence of betadine, the decrease in *V. indigofera* and increase in *P. putida* concentrations in binary biofilms was significant under all growth conditions (Table 4, Fig. 3). The differential response of *V. indigofera* and *P. putida* to mono- and binary culture in the presence and absence of betadine indicated a significant interaction between these variables, i.e. each species responded differently to binary culture conditions and to betadine disinfection.

Discussion

Effects of community composition and dilution rate on total biofilm populations

Biofilms formed from binary and 20-isolate planktonic communities produced denser biofilms (CFU cm^{-2}) than monocultures at both DRs. Increased species diversity may provide spatial and temporal niches within the biofilms not available within monocultures or may create microenvironments within the biofilm (Korber *et al.*, 1993; Lens *et al.*, 1993; de Beer *et al.*, 1994a). The 20-isolate community may also contain one or more members that are particularly adept at biofilm colonization in mixed culture. Piliation of *P. putida*, which can facilitate adhesion to surfaces, can certainly account in part for its dominance in biofilm populations. Also, interspecies synergistic interactions involving adhesion targets, metabolites or quorum sensing (McLean *et al.*, 1997) may provide bacterial microenvironments more conducive or attractive

Table 3. Effect of community composition, betadine concentration and dilution rate on biofilm survival[a] as determined by three-way ANOVA.

Dilution rate effects		
	Survival[a]	
Community composition	0.02% Betadine	0.04% Betadine
Pseudomonas putida	0.0083 h^{-1} = 0.033 h^{-1} NS[b]	0.0083 h^{-1} > 0.033 h^{-1}*
Vogesella indigofera	0.0083 h^{-1} < 0.033 h^{-1}**	0.0083 h^{-1} < 0.033 h^{-1}**
Binary	0.0083 h^{-1} = 0.033 h^{-1} NS	0.0083 h^{-1} < 0.033 h^{-1}*
Twenty strain	0.0083 h^{-1} < 0.033 h^{-1}***	0.0083 h^{-1} < 0.033 h^{-1}***

Community comparisons[c]			
DR = 0.0083 h^{-1}		DR = 0.033 h^{-1}	
0.02% Betadine	0.04% Betadine	0.02% Betadine	0.04% Betadine
V > B*	V > B NS	V > B**	V > B*
V > 20***	V > 20**	V > 20***	V = 20 NS
V > Ps***	V > Ps**	V > Ps***	V > Ps***
B > 20*	B > 20***	B = 20 NS	B = 20 NS
B = Ps NS	B > Ps***	B > Ps***	B > Ps***
20 = Ps NS	20 = Ps NS	20 > Ps***	20 > Ps***

a. Survival calculations outlined in text.
b. Significance denoted by *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, no significant difference at $P = 0.05$.
c. Community descriptions: V, *Vogesella indigofera*; Ps, *Pseudomonas putida*; B, binary culture; 20, 20 strain culture.

Table 4. Influence of dilution rate, betadine concentration and community growth on *Pseudomonas putida* and *Vogesella indigofera* interactions in binary biofilm populations as determined by three-way ANOVA.

Betadine concentration	Dilution rate	Community[a]	Significance[b]
Pseudomonas putida			
0%	0.0083 h^{-1}	B > M	*
0	0.033 h^{-1}	B > M	NS
0.02	0.0083 h^{-1}	B > M	***
0.02	0.033 h^{-1}	B > M	***
0.04	0.0083 h^{-1}	B > M	*
0.04	0.033 h^{-1}	B > M	**
Vogesella indigofera			
0%	0.0083 h^{-1}	B < M	*
0	0.033 h^{-1}	B < M	NS
0.02	0.0083 h^{-1}	B < M	*
0.02	0.033 h^{-1}	B < M	***
0.04	0.0083 h^{-1}	B < M	*
0.04	0.033 h^{-1}	B < M	*

Component populations Betadine concentration	Dilution rate	Community[a]	Populations[c]	Significance[b]
0%	0.0083 h^{-1}	M	V > Ps	*
0	0.0083 h^{-1}	B	Ps > V	**
0	0.033 h^{-1}	M	Ps > V	NS
0	0.033 h^{-1}	B	Ps > V	**
0.02	0.0083 h^{-1}	M	V > Ps	***
0.02	0.0083 h^{-1}	B	Ps > V	NS
0.02	0.033 h^{-1}	M	V > Ps	***
0.02	0.033 h^{-1}	B	Ps > V	NS
0.04	0.0083 h^{-1}	M	Ps = V	NS
0.04	0.0083 h^{-1}	B	Ps > V	**
0.04	0.033 h^{-1}	M	V > Ps	***
0.04	0.033 h^{-1}	B	Ps > V	NS

a. Communities designated as monoculture (M) and binary (B) culture.
b. Significance denoted by *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, no significant difference at $P = 0.05$.
c. Population descriptions: V, *Vogesella indigofera*; Ps, *Pseudomonas putida*.

to bacterial colonization and thus may serve to increase bacterial numbers in mixed culture biofilms.

Nutrient availability, controlled here by DR, strongly affected total biofilm population density, as shown in Table 2. In general, we found higher numbers of bacteria attached to discs within the Modified Robbins' Device (MRD) at a DR of 0.0083 h^{-1} than at a DR of 0.033 h^{-1}. Thus, biofilm population sizes appear to be significantly affected by carbon availability, with higher numbers of bacteria present in biofilms under increasing carbon limitation. Our data support the concept of biofilm formation being promoted as a starvation response in oligotrophic environments (Dawson *et al*., 1981). Possible explanations for this response include increased nutrient affinity in biofilm bacteria (Fletcher, 1986; Prigent-Combaret *et al*., 1999) compared with planktonic bacteria and the concentration of nutrients on surfaces due to interfacial effects (McLean and Beveridge, 1990). We did not measure available glucose concentrations within the chemostat, which may also affect biofilm growth. Sampling at different times was not addressed in this study because of technical difficulties. The MRD has 25 sample plugs and could conceivably be used to collect biofilm samples at various time points. It was our experience that repeated sampling from a MRD resulted in significant contamination. Nevertheless, further studies, particularly at varying time points, are required to identify the mechanisms involved in the observed effect of carbon limitation on biofilm formation.

The significant interaction of community composition and DR on total biofilm numbers detected by analysis of variance (ANOVA) indicates that community composition effects on biofilm numbers are complex and dependent on the DR. Nutrition and growth rate both affect bacterial physiology, with the impact varying among species (Kolter *et al*., 1993). On this basis, one would anticipate that species' interactions, based in part on physiology, would also be affected by DR. Although biofilm numbers decreased for all communities at the higher DR, the magnitude of the decline differed between communities. As illustrated in Fig. 1, monocultures of *V. indigofera* and 20-isolate biofilm communities showed the largest rate of reduction at DR 0.033 h^{-1}. Thus, although biofilm numbers are affected by both DR and community composition, the interaction between these variables may be equally important in determining biofilm numbers. This result emphasizes the importance of analysing natural biofilm communities in which bacterial diversity and nutrient concentrations are varied.

Biofilm susceptibility to betadine

As expected, betadine concentration affected survival (Table 3). With the exception of the *P. putida* monoculture community, the other biofilms formed at DR 0.033 h^{-1} were more resistant overall to 0.04% iodine disinfection than biofilms grown at DR 0.0083 h^{-1}. When 0.02% betadine was used, significant differences due to DR were seen only in the *V. indigofera* and 20-strain biofilms. Although numbers of bacteria in biofilms were greater at the lower DR, these biofilms were generally more susceptible to betadine disinfection. This contrasts with speculation that decreased susceptibility to biocides is due to differences in physiology associated with lower growth rates (Brown and Gilbert, 1993). Enhanced betadine resistance at higher growth rates may be explained by reduced diffusion of the biocide into the biofilm (de Beer *et al*., 1994b). Although not tested in this study, one possible explanation is that biofilms grown at the higher DR produce higher quantities of extracellular polymers and can thus reduce iodine diffusion into the biofilm. Alternatively, one or more organisms may be able to degrade betadine, a feature enhanced at a higher DR. *P. putida* monocultures did not exhibit increased survival at the higher DR, indicating that DR did not have the same effect on each biofilm population. Thus, although susceptibility to betadine may be influenced by DR, this influence could also be correlated with the inherent resistance of the community.

When compared with monocultures, the higher population numbers of binary and 20-isolate biofilms did not lead to enhanced survival to betadine disinfection (Table 3). As higher biofilm populations at DR 0.0083 h^{-1} also exhibit high betadine susceptibility, these results suggest that survival of biofilm bacteria was independent of population numbers of the biofilms. This finding is in contrast to that reported by Brown and Gauthier (1993), who found that cell density was important in establishing resistance to betadine. Our study evaluated resistance in biofilms of statistically different initial numbers, but the maximum difference was one order of magnitude. It is possible that cell density is an important factor when evaluated over a wide range of densities. Our study also involved growth of natural isolates (Table 1) grown under carbon limitation in defined medium rather than a laboratory strain of *P. aeruginosa* grown in complex medium (nutrient broth and glucose). In addition, we studied bacterial colonization and biofilm formation on a sterile silicon surface compared with growth of a filter-concentrated planktonic population (artificial biofilm) of *P. aeruginosa* (Brown and Gauthier, 1993). Biofilms formed in our study may have developed natural structures such as microcolonies and water channels (Lawrence *et al*., 1991) that are not present in artificial biofilms. Iodine resistance, noted in the present study, may arise partially from iodine entrapment inside the biofilm matrix (Van der Wende *et al*., 1989) and thus may be a function of the physiology of individual species.

Although DR and community composition affected

numbers of biofilm bacteria, our disinfection studies indicate that larger population numbers do not provide increased resistance to betadine. In fact, under conditions in which more bacteria inhabited biofilms, i.e. DR 0.0083 h^{-1} and 20-isolate communities, the survival rate was shown to decrease (Fig. 4). Thus, an increase in biofilm population by two orders of magnitude or less may not provide larger numbers of survivors after disinfection. Instead, survival may depend predominately on the growth rate and intrinsic resistance of the biofilm constituents.

Interactions of V. indigofera *and* P. putida *in binary biofilms*

The relative concentrations of *P. putida* and *V. indigofera* in binary biofilms could not be predicted from their relative abundance in the planktonic community. The overall effect of growth condition (monoculture or binary) on population size, as determined by ANOVA, was shown to be significant, indicating interactions between *P. putida* and *V. indigofera* in binary culture. If no interactions were occurring between these species, binary biofilm numbers would equal the sum of *P. putida* and *V. indigofera* monoculture biofilms for each DR. Differences between $\log_{10}$ expected and measured total binary numbers are small, with expected $\log_{10}$ population values of 7.12 (measured value 7.02) and 6.86 (measured value 6.83) for DR 0.0083 h^{-1} and 0.033 h^{-1} respectively. However, Fig. 3 clearly indicates that binary biofilms are not simply a result of the addition of monoculture values. Instead, *P. putida* has a negative effect on *V. indigofera* populations. This negative interaction may be partially explained by the differences in attachment rates of *P. putida* and *V. indigofera*. *P. putida* was shown to have a much higher rate of attachment over the first 3 h (2.6×10^4 CFU cm^{-2}) than *V. indigofera* (2.6×10^1 CFU cm^{-2}), and this initial rate of adhesion may provide *P. putida* with an initial advantage in biofilm formation. The number of *P. putida* in biofilms did not increase significantly when grown in binary culture, whereas *V. indigofera* showed decreases in biofilm numbers in binary culture with the decrease at DR 0.0083 h^{-1} being highly significant. Thus, the largest decrease in *V. indigofera* numbers in binary culture occurred when *V. indigofera* was at its highest percentage (48%) in the planktonic population. This observation once again reinforces the concept that planktonic population composition may not accurately predict the biofilm population (James *et al*., 1995).

Susceptibility of binary biofilms to betadine

Mono- and binary cultures of *P. putida* and *V. indigofera* responded differently to betadine disinfection (Fig. 4, Table 3). *P. putida* benefited from the presence of *V. indigofera*, a more betadine-resistant species in binary biofilms. The converse was not true. This observation provides evidence for cell–cell shielding within the binary biofilm. *V. indigofera* was more resistant to betadine disinfection and this resistance may have facilitated survival of the more susceptible species, *P. putida*. As growth rate had no significant effect on individual species survival, this finding provides evidence that the species composition of binary biofilms is important in determining survival of component species to betadine disinfection. A number of mixed population studies with biofilms have been conducted previously. *Pseudomonas fluorescens* has been shown to protect *Salmonella typhimurium* against chlorine (Leriche and Carpentier, 1995). Coliform survival is enhanced in mixed population biofilms (Camper *et al*., 1996). Several studies have shown the impact of nutrition and antimicrobial agents on biofilms (Costerton *et al*., 1995; Kim and Frank, 1995). The majority of these studies have investigated one or two parameters, typically total bacterial concentrations or the concentration of one particular organism under a single growth condition. In the present study, we have examined the interaction of several factors – growth rate, community composition and (with binary cultures) the interactions of two species (*P. putida* and *V. indigofera*) on biofilm and planktonic populations – and the influence of these factors on biofilm resistance to betadine disinfection. Of particular note, the biofilm formation and betadine resistance was influenced significantly by community composition and growth rate. Therefore, resistant bacterial components of mixed culture biofilms may protect more susceptible bacteria, including pathogens, against betadine disinfection, again emphasizing the importance of studying the efficacy of disinfectants in mixed culture biofilms.

Experimental procedures

Collection, isolation and identification of bacteria

Naturally occurring sessile and planktonic bacteria were isolated from the Edwards Aquifer, a karst aquifer in south central Texas, USA, described by Longley (1994). Rock-adherent, sessile bacteria were collected by first placing sterilized 3- to 5-cm-diameter limestone rocks (autoclaved at 121°C for 15 min) into sterile fibreglass screen bags and then aseptically suspending the bags in the underground waters of the Edwards Aquifer through an access cave on the Southwest Texas State University (SWT) campus. After 7 days, the rocks were aseptically removed and transported to the laboratory. Planktonic bacteria were isolated from water samples aseptically collected from the same access cave.

Bacterial isolates were obtained on R2A (Difco) medium and each was grown in pure culture to late log phase in minimal salts medium for 48 h at 25°C with glucose (0.25 g l^{-1}) (Whiteley *et al*., 1997). Glycerol was then added at a concentration of 12.5% (v/v) and the culture

suspension stored at −80°C (McLean *et al*., 1999). Thirteen species were identified from these isolates on the basis of published characteristics (Krieg and Holt, 1984) using microscopic analysis and biochemical tests including the Biolog Identification System (Biolog) (Amy *et al*., 1992) (Table 1). Identifications for all species were confirmed by the Biolog Identification System on the basis of similarity index values of ≥ 0.50. The identities of the most common planktonic bacteria, *V. indigofera*, subsequently grown in chemostat culture and the most common surface-adherent bacteria, *P. putida*, subsequently grown in a MRD were confirmed by sequencing the 16S rRNA fraction from each species (Reeves *et al*., 1995).

Culture types (communities)

We examined monocultures of *P. putida* and *V. indigofera*, a binary culture of *P. putida* and *V. indigofera*, and a mixed culture (community) of 20 strains including *P. putida* and *V. indigofera*. In initial experiments involving mixed continuous culture of 20 strains, *P. putida* and *V. indigofera* represented the most common sessile and planktonic species respectively. Thus, these species were selected for monoculture and binary cultures. Both species were readily identifiable on the basis of colony pigmentation on R2A medium.

Growth conditions and generation of microbial biofilms

The general strategy and apparatus used for chemostat culture of biofilms has been described previously (Whiteley *et al*., 1997; McLean *et al*., 1999). Before each experiment, cultures were retrieved from frozen stock and streaked onto R2A agar to check for purity. Well-isolated colonies were then used to inoculate minimal medium supplemented with glucose (Whiteley *et al*., 1997). Planktonic cultures were formed by suspending 0.5 ml of 0.2 OD_{600} of freshly grown cultures of each community member into a sterilized chemostat. To allow the organisms to become established, the chemostat was first maintained under batch conditions for 24 h at 25°C before continuous culture was established. Each culture type was then grown at DRs of 0.0083 h^{-1} and 0.033 h^{-1} in minimal salts medium with glucose as the sole carbon source (0.25 g l^{-1}). Numbers of planktonic bacteria within the chemostat were monitored daily to ensure stabilization of planktonic communities and monitor contamination. To investigate the fate of individual species in binary culture, the relative numbers of *P. putida* and *V. indigofera* were measured as described above. After the planktonic populations had stabilized for 6 days, a MRD (Tyler Research) (McLean *et al*., 1999), containing silicon discs, was attached to the chemostat and biofilms were allowed to form for 24 h. In this fashion, we could grow biofilms from physiologically stable, known bacterial communities that had been originally isolated from natural biofilm populations.

Biofilm sampling and disinfection with betadine

After 24 h, 15 silicon discs were selected at random and removed from the MRD. Discs were dipped in 10 ml sterile phosphate-buffered saline (PBS) for 1 min to remove planktonic and loosely attached cells. CFUs (expressed as CFU cm^{-2}) were measured on five discs and the mean number of CFUs of these five discs was used to estimate the average number of bacteria before betadine treatment (N_0). Five discs were subjected to a 0.04% (v/v) betadine dilution, and five were subjected to a 0.02% (v/v) betadine dilution. After betadine treatment, the CFU cm^{-2} was measured on each of the five discs in each treatment (N), and the mean $\log_{10}$ CFU cm^{-2} of these discs was used to estimate the mean number of bacteria after the respective betadine treatment. Survival after betadine treatments was estimated as $\log_{10}(N/N_0)$.

To test the susceptibility of biofilm bacteria in each of the four cultures to betadine disinfection, each disc was exposed, biofilm side down, to 10 ml of a 0.02% or 0.04% (v/v) dilution of betadine (10% (w/v) polyvinylpyrrolidone iodine if undiluted) for 3 min in sterile Petri dishes. Preliminary experiments showed that a 3 min exposure to 0.02% betadine reduced culturable planktonics from 10^8 to $< 10^2$ for the most resistant culture (*V. indigofera*) and from 10^8 to $< 10^1$ for the other three cultures. After betadine exposure, all silicon discs were aseptically transferred into 10 ml of sterile 5% (w/v) sodium thiosulphate for 3 min to inactivate residual betadine. Thiosulphate treatment did not affect bacterial counts (data not shown). All discs were aseptically removed from the sodium thiosulphate and placed into sterile PBS, pH 7.2, to enumerate bacteria.

Bacterial enumeration

To enumerate bacteria, biofilm-coated discs were suspended in 3 ml PBS, sonicated in a 60 Hz bath sonicator (Sonicor Instruments) at 22°C for 5 min, then vortexed vigorously for 3 min to remove and disseminate biofilm bacteria. These times provided maximum retrieval of biofilm bacteria (data not shown). Suspended bacteria were then serially diluted in sterile PBS and plated onto R2A agar. Plates were incubated at 25°C for 48 h and CFU cm^{-2} determined. For binary cultures, we determined total bacterial counts as well as the numbers of *P. putida* and *V. indigofera*.

Statistical analysis

Four replicates were performed for each community, DR and betadine combination. Overall means for each combination of community, DR and betadine concentration were computed as the average of the means of each of the four replicates. Variation in $\log_{10}$ total biofilm population numbers (CFU cm^{-2}) of the four communities at DRs of 0.0083 h^{-1} and 0.033 h^{-1} was analysed using two-way ANOVA with community composition and DR considered as fixed effects. Means were compared between communities within each DR and between DRs for each community using Ryan's Q at alpha = 0.05 (Day and Quinn, 1989). To assess the effects of community composition, betadine, DR and the interactions of these effects on survival of biofilm bacteria, a three-way ANOVA followed by means comparisons was used. In no case did arcsin square root transformation of survival data improve model fit, so the analysis reported herein was conducted on

$\log_{10}$ (N/N_0) survival values. The statistical analysis was performed using the mainframe version 6.09 of the Statistical Analysis System (SAS Institute).

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