Microbial colonization and biodeterioration of plasticised polyvinyl chloride

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TABLE OF CONTENTS

Abstract

ABSTRACT

Major problems of substratum damage can occur when plasticised polyvinyl chloride (pPVC) is colonised by microorganisms. In order to develop new strategies to control biodeterioration of pPVC, knowledge of the colonizing organisms and their interactions with the substratum is essential. This study therefore aimed i) to investigate microbial colonization processes occurring on pPVC *in-situ*, ii) to study the mechanism of adhesion of important colonizing organisms to pPVC, and iii) to develop novel techniques to compare the efficacy of biocides against the relevant deteriogenic organisms.

The microbial colonization of pPVC was investigated *in-situ* by exposing pPVC to the atmosphere in a 2 year longitudinal experiment. Fungal and bacterial populations were quantified, and colonizing fungi were identified with rDNA sequence and morphological characters. *Aureobasidium pullulans* was the principal colonizing fungus, establishing on the pPVC after 25 weeks of exposure. A group of yeasts and yeast-like fungi, including *Rhodotorula aurantica* and *Kluyveromyces spp.*, established much later (after 80 weeks). No bacterial colonization was observed. *In-vitro* tests were used to characterise the deteriogenic properties of fungi isolated from the pPVC. All strains of *A. pullulans* tested could grow with the intact pPVC formulation as the sole source of carbon and could degrade the plasticiser dioctyl adipate (DOA). In contrast, several yeast isolates could not grow on pPVC or degrade DOA. These results suggest that microbial succession may occur during the colonization of pPVC and highlight the importance of *A. pullulans* in the establishment of a microbial community on pPVC.

A quantitative adhesion assay using image analysis was developed to investigate the mechanism of adhesion of *A. pullulans* to pPVC containing the plasticisers DOA and dioctyl phthalate (DOP). Adhesion to unplasticised PVC (uPVC) was also monitored as a control. Adhesion to pPVC was found to be greater than to uPVC by a maximum of 280% after a 4 h incubation using 1.0×10^8 blastospores ml^{-1}. The adhesion assay also identified fundamental differences in the mechanism of adhesion to the two materials. Adhesion to uPVC was primarily controlled by hydrophobic interactions and was not influenced by the electrostatic properties of the suspension buffer (i.e. pH and electrolyte concentration). In contrast, adhesion to pPVC was strongly influenced by electrostatic interactions, and was optimal in the pH range 6 to 10 and with an electrolyte concentration of 10 mM NaCl. The plasticisers DOP and DOA therefore

increase adhesion of *A. pullulans* to PVC through an interaction that is mediated by electrostatic forces.

Expression of green fluorescent protein (GFP) in *A. pullulans* was investigated both as a marker system to study the colonization of pPVC, and as a potential reporter of the susceptibility of the fungus to antimicrobial compounds. The transformed strain Ap1*gfp* was readily observed on pPVC using epifluorescence microscopy and was amenable to quantification in real-time using image analysis. To investigate the possible use of GFP as an indicator of antimicrobial susceptibility, a range concentrations of each of the biocides sodium hypochlorite and 2-*n*-octylisothiazolin-3-one (OIT) to suspensions of Ap1 *gfp* blastospores (pH 5 buffer). These biocides each caused a rapid (< 25 min) loss of fluorescence of greater than 90% when used at concentrations of 150 μg ml^{-1} available chlorine and 500 μg ml^{-1} respectively. Furthermore, loss of GFP fluorescence from *A. pullulans* cells was highly correlated with a decrease in the number of viable cells ($R^2 > 0.92$). This technique allowed the relative antimicrobial properties of a range of biocides to be compared using simple fluorescence measurements and should have broad applications in testing the susceptibility of *A. pullulans* and other fungal species to antimicrobial compounds.

DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other institute of learning.

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THESIS ORGANISATION AND STRUCTURE

The results chapters within this thesis are presented in the style of the international peer-reviewed journal *Applied and Environmental Microbiology*. Chapters 2, 3, and 5 are published or have been submitted for publication within this journal as follows:

Chapter 2: Webb, J. S., M. Nixon, I. M. Eastwood, M. Greenhalgh, G. D. Robson, and P. S. Handley. 2000. Fungal colonization and biodeterioration of plasticised polyvinyl chloride. *Applied and Environmental Microbiology*. In press.

Chapter 3: Webb, J. S., H. C. Van der Mei, M. Nixon, I. M. Eastwood, M. Greenhalgh, S. J. Read, G. D. Robson, and P. S. Handley. 1999. Plasticisers increase adhesion of the deteriogenic fungus *Aureobasidium pullulans* to polyvinyl chloride. *Applied and Environmental Microbiology* 65:3575-3581

Chapter 5: Webb, J. S., S. R. Barratt, M. Nixon, I. M. Eastwood, M. Greenhalgh, G. D. Robson, and P. S. Handley. GFP as a novel indicator of antimicrobial susceptibility in *Aureobasidium pullulans*. *Applied and Environmental Microbiology*. Submitted.

All of these chapters therefore stand alone and contain their own figure and table numbers and list of references. A consequence of this is that there is inevitably minor overlap and repetition in methodology between chapters. Spelling has also been americanised throughout these chapters. Because of the brevity in methodology required for journal publications, some methods are expanded further within the appendices. Results of some preliminary studies that did not fall within the scope of these papers are also presented within the appendices.

Authors Dr. Pauline S. Handley and Dr. G. D. Robson are supervisors of this project at the University of Manchester. Dr I. M. Eastwood, Malcolm Greenhalgh and Marianne Nixon (Avecia Biocides, Manchester) are joint industrial CASE supervisors. Measurement of the electrostatic properties of *Aureobasidium pullulans* cells (Chapter 3, Fig. 7) was conducted by Dr. Henny C. van der Mei, Department of Biomedical Engineering, University of Groningen, The Netherlands. Studies of GFP fluorescence and cell viability in *A. pullulans* (Chapter 5) were carried out with assistance from Sarah R. Barratt, a PhD student at the University of Manchester.

All other practical studies, and all of the written text in this thesis is the work of JSW.

For Mum, Dad
and Jessica

"If someone has mould sent upon their house, then they must go and tell the priest about it. If the priest sees spots that appear to be eating the wall, he shall leave the house and lock it for seven days. On the seventh day he shall return and examine it again. If the mould has spread he must have all the walls scraped and the material dumped in an unclean place outside the city... If the mould breaks out again, the house is unclean. It must be torn down, and its stones, its wood and its plaster must be carried out of the city to an unclean place".

LEVITICUS 14, 34-48
Old Testmt.

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ABBREVIATIONS

Ap1 *gfp*	transformed *Aureobasidium pullulans* strain (PRAFS8)
BBIT	n-butyl-1,2-benzisothiazolin-3-one
CFU	colony forming unit
DAPI	4'-6'-diamidino-2-phenylindole
DCA	dynamic contact angle analyser
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide triphosphate
DOA	dioctyl adipate
DOP	dioctyl phthalate
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EPS	extracellular polymeric substances
FACS	fluorescence-activated cell sorting
FEP	fluoroethylene polypropylene
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
ITS	internal transcribed spacer
LTSEM	low temperature scanning electron microscopy
MEA	malt extract agar
MSM	mineral salts medium
MSYE	mineral salts medium supplemented with yeast extract
NCMP	n-(trichloromethylthio)phthalimide
OBPA	10,10'-oxybisphenoxyarsine
OIT	2-n-octyl-4-isothiazolin-3-one
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDA	potato-dextrose agar
PE	polyethylene
PET	polyethylene tetraphthalate
PNB	p-nitrophenyl butyrate
PP	polypropylene
pPVC	plasticised polyvinyl chloride
PTFE	polytetrafluoroethylene
R^2	correlation coefficient
REE	relative extracellular esterase activity unit
ROI	region of interest
SEM	scanning electron microscopy
TCMP	2,3,5,6-tetrachloro-4-(methylsulphonyl)pyridine
TC-PET	tissue culture-treated polyethylene tetraphthalate
TEF	translation elongation factor
uPVC	unplasticised polyvinyl chloride

Chapter 1.

General introduction

GENERAL INTRODUCTION

The production of plastics worldwide exceeds 100 million metric tons per year (Elias, 1992). However, the action of microorganisms can severely damage or limit the useful life of many synthetic polymers and causes vast industrial and medical problems (Eastwood, 1994; Morton & Surman, 1994; Flemming, 1998). Polyurethanes, polyesters, celluloses and plasticised PVC (pPVC) are all examples of plastic products that are susceptible to microbial attack. In the case of pPVC, biocides are frequently incorporated within the plastic to control microbial surface growth. This biocides market is an estimated £60 million in the U.K. (Eastwood, I. M., pers. comm.). However, increasing regulatory requirements and environmental concerns mean that there is a continual need to develop new strategies and biocides to prevent microbial damage to pPVC. For the successful development of these technologies it is essential to understand the microbial colonization process that occurs on the substratum. This study therefore aimed to determine which are the important organisms that cause biodeterioration of pPVC, how they interact with the surface, and to establish techniques to compare the efficacy of biocides against the relevant organisms.

BIODETERIORATION of pPVC

Biofilms and Biodeterioration.

The vast majority of microorganisms within nature are found attached to surfaces within biofilms (Costerton *et al.*, 1987). Biofilms may consist of complex communities of bacteria, fungi, algae and protozoa which display morphological and physiological adaptations to surface growth that are not observed in the free-living organisms. Through a combination of their physical presence and metabolic activity, biofilms usually cause damage to the surface or result in obstruction so that the efficiency of the surface is reduced. Any undesirable change in the properties of the material caused by the presence and activity of a biofilm may be described as **biodeterioration** (Morton & Surman, 1994).

The formation of bacterial biofilms at the solid-liquid interface has been extensively researched (for a review, see Stickler, 1999). Attachment of bacteria to surfaces is typically followed by the production of stack-like microcolonies that are separated by water channels and embedded within an organic polymer matrix (Keevil *et al.*, 1993;

Costerton *et al.*, 1994). This mode of growth confers a number of advantages on microorganisms within a biofilm. Sessile cells may benefit from increased nutrient availability because the biofilm concentrates inorganic and organic molecules from the environment. The surface itself may become a source of nutrients for microorganisms, as in the case of plasticised PVC, where additives to the PVC formulation can act as a carbon source for microbial growth (Griffin & Uribe, 1984). In addition cells in a biofilm may gain increased protection from antimicrobial agents. Both bacteria (Keevil *et al.*, 1990; Surman *et al.*, 1995) and fungi (Baillie & Douglas, 1998) within a biofilm have demonstrated increased resistance to antimicrobial compounds relative to those in the planktonic phase. This resistance may be due to the protection conferred by extracellular polymeric substances (EPS) produced by a biofilm (Costerton *et al.*, 1981) or due to physiological changes in response to surface attachment, such as changes in growth rate (Evans *et al.*, 1990). A review of the problems associated with biocide resistance within biofilms is provided by Morton *et al.*, (1998). The proximity of cells within a biofilm may also promote genetic exchange between cells. Cells may therefore acquire characteristics that provide a selective advantage (Bale *et al.*, 1988). Microbial attachment to surfaces can therefore be regarded as a general survival strategy in low nutrient and hostile environments.

While many studies have examined bacterial biofilms in aqueous systems, few studies have examined biofilms that form at solid/air interfaces. Biodeterioration of pPVC exposed to the atmosphere in terrestrial environments is an example of such a biofilm, where fungi are reported to be the predominant colonizing organisms (Bessems, 1988; Hamilton, 1983). The interactions that occur between microorganisms and the substratum at solid/air interfaces are poorly understood. Furthermore, although fungi are recognised as excellent colonizers of surfaces (Jones, 1992), the study of fungal biofilms is a new field of research. Recent studies have shown that fungi, like bacteria, may produce differentiated biofilm phenotypes that are distinct from the planktonic form. For example, *Candida albicans* biofilms produce an EPS matrix that is absent from planktonic cells (Hawser *et al.*, 1998). They also show increased resistance to antifungal agents (Baillie & Douglas, 1998).

Plasticised PVC.

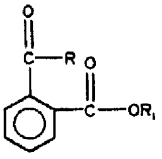
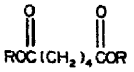
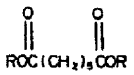
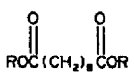
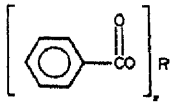
Plasticised PVC is used in an extensive range of industrial and domestic applications, for example swimming pool liners, roofing and tunnel membranes, electrical cable insulation,

inflatable buildings, wall and floor coverings and shower curtains (Lorenz, 1990). pPVC is poly(vinyl chloride) resin that has been modified by the addition of plasticisers and other additives so that the normally hard, rigid PVC resin becomes a flexible, extensible and resilient polymer. pPVC is an extremely versatile plastic; variation in the choice of additives and plasticisers allows the physical, chemical and electrical properties of pPVC to be tailored to a broad range of applications.

The basic PVC polymer, $(CH_2CHCl)n$, forms the strong but rigid unplasticised PVC (uPVC), applied for example in the production of window frames and pipe materials. Incorporation of plasticisers allows the resin molecules to flow over one another, producing the highly flexible yet strong material, pPVC. Each year, Western Europe uses approximately one million tonnes of plasticisers with a value in the order of £1, 000 M (Wilson, 1995). Some of the common chemical classes of plasticisers are listed in Table 1. In order to be compatible with PVC, plasticisers must be of sufficient polarity to interact with the PVC by hydrogen bonding (Meier, 1990). If incompatible, the plasticiser may be exuded from the polymer and migrate into the material with which it comes into contact.

The pPVC used in this study contained two plasticisers, di-octyl phthalate (DOP) and di-octyl adipate (DOA), in equal proportions. Together, these plasticisers constitute approximately 32% of the mass of the plastic. In addition to plasticisers, a number of other additives were incorporated in the formulation. The pPVC included small amounts

Table 1. Common groups of plasticisers used in PVC (from Seymour, 1992).

General type	Structure	Examples
Phthalate esters		R = R_1 = 2-ethylhexyl, methyl, ethyl, butyl, cyclohexyl; R = butyl, R_1 = benzyl
Phosphate esters	$(RO)_3PO$	R = ethyl, phenyl, cresyl
Adipate esters		R = butyl, 2-ethylhexyl
Azelate esters		R = butyl, 2-ethylhexyl
Sebacate esters		R = butyl
Benzoate esters		R = neopentyl ($x = 2$), glyceryl ($x = 3$)

of stearic acid and zinc stearate which act as lubricants to aid processing of the plastic. Fatty acids and their salts are added to reduce friction between the plastic and metal during calendering and mold filling (Coran & Lederer, 1992). In addition, the pPVC contained the white pigment TiO_2. As well as colouring the plastic white, this compound also provides protection against ultraviolet (UV) degradation. UV degradation involves mainly sunlight and oxygen and causes deterioration of physical properties and appearance of the pPVC (Hollande & Laurent, 1997). Pigments such as TiO_2 prevent UV degradation of the pPVC by blocking light from the resin and plasticisers. Photostabilizing additives such as amines, benzoates or phosphites may also be used to prevent UV degradation.

PVC resin is itself biologically inert (Griffin & Uribe, 1984). However, many of the additives incorporated within pPVC can be degraded by microorganisms. Of these, the plasticiser is the major contributor to microbial susceptibility (Tirpak, 1970).

Microbial degradation of plasticisers.

Microbial degradation of plasticisers results in brittleness, shrinkage, cracking and ultimately failure of the PVC in its intended application. Plasticisers vary in their susceptibility to microbial attack. This was first demonstrated by Berk *et al.* (1957). A total of 127 different plasticisers and plasticiser-related compounds were evaluated for their ability to serve as nutrient sources for 24 separate fungi in 13 different genera. The relative susceptibility of the various groups of plasticisers are shown in Table 2. Bacterial degradation of plasticisers has also been demonstrated. Booth *et al.*, (1968) and Booth & Robb (1968) found species of *Pseudomonas, Achromobacter, Brevibacterium, Nocardia, and Bacillus* to be able to use plasticisers as nutrients. Eaton & Ribbons (1982) isolated several strains of *Micrococcus* by enrichment on phthalate esters as the sole carbon source, and Burgess & Darby (1965) reported substantial shrinkage associated with the removal of sebacate plasticisers from pPVC by *Pseudomonas aeruginosa*. From these and subsequent studies, plasticisers have become grouped according to their susceptibility to microorganisms. Plasticisers with little or no susceptibility include phthalates, phosphates and sulfonates whereas those with medium to high susceptibility include adipates, sebacates, epoxidised fatty acids, oleates, stearates and polyesters (Lorenz, 1990). The susceptibility of the same plasticisers within commercial formulations, however, can vary greatly. For example, polyadipate

Table 2. Ability of various kinds of plasticisers to support growth of fungi. Plasticisers were homogenised in mineral salts agar and inoculated with a range of 24 separate fungi. A relative susceptibility rating was derived for each plasticiser by averaging colony diameters for all of the fungi (From Berk *et al.*, 1957)

Plasticiser group	Average colony diameter (cm)
Ricinoleic acid esters	6.1
Oleic acid esters	5.9
Polyesters	5.6
Azelaic acid esters	3.9
Sebacic acid esters	3.8
Succinic acid esters	2.8
Esters of polyols	2.7
Adipic acid esters	2.2
Glycollic acid esters	0.6
Phthalic acid esters	0.2
Maleic acid esters	0.1
Phosphorid acid esters	0.0
Dextrose control	6.3
Glycerol control	6.0

plasticisers previously shown to support considerable fungal growth when homogenized in agar, were resistant to fungal and bacterial attack when incorporated into pPVC strips (Klausmeier & Jamison, 1973). Such anomalies can be explained by considering the mobility of the plasticisers within the formulation. Since microbial activity is limited to the surface of the plastic, plasticisers that do not readily migrate throughout structure of the polymer will be less susceptible to attack.

Biochemistry of plasticiser degradation

The majority of plasticisers are organic esters (Seymour, 1992). Thus early assumptions were made that the deterioration of these compounds was initiated by esterase enzymes. Berk *et al.* (1957), for example, concluded that clear zones around organisms growing on plasticiser agar were caused by extracellular esterases diffusing beyond the colony and converting insoluble esters into water-soluble components. Subsequent studies have supported these assumptions. Mycelia of organisms capable of degrading plasticisers have been shown to contain esterases capable of degrading dibutyl sebacate (Mills & Eggins, 1974). Klausmeier & Jones (1961) have identified the production of monopropyl phthalate from fungi grown on dipropyl phthalate-containing medium, confirming esterolytic cleavage. Extracellular esterase enzymes have also been demonstrated to

cause degradation of polyester polyurethanes, which contain many ester bonds (Akutsu *et al.*, 1998; Vega *et al.*, 1999).

Once a plasticiser has been de-esterified the biochemistry of its catabolism is well understood. The resulting fatty acids, dicarboxylic acids, and alcohols are all degraded by microorganisms through well established metabolic pathways. In the case of aromatic phthalate plasticisers, cleavage of the aromatic ring may subsequently occur. The initial stage in the degradation of the benzene ring is the formation of either of two molecules, protocatechuate or catechol (Bugg & Winfield, 1998). These single ring compounds are referred to as starting substrates because oxidative catabolism proceeds only after the complex aromatic molecules have been converted to these more simple forms. Protocatechuate and catechol may then be further degraded to compounds that enter the citric acid cycle. Metabolic pathways for the complete catabolism of phthalate esters by various *Micrococcus spp.* have been identified by Eaton & Ribbons, (1982). The majority of catabolic routes found among *Micrococcus* species progress via protocatechuate and subsequent ortho- or meta- cleavage of the aromatic ring structure.

Co-metabolism of plasticisers

Plastics in use are frequently contaminated with organic matter that will support microbial growth. Bacteria and fungi have been isolated from the environment that can degrade many commercial plasticisers but only in the presence of extraneous organic nutrients (Klausmeier, 1966). Plasticisers were homogenised in yeast-extract agar and those colonies surrounded by zones of clearing were selected for further study. 69% of bacteria and 16% of fungi were capable of degrading plasticisers only when yeast extract was present in the medium, i.e. demonstrated co-metabolism. The study showed that the percentage of organisms capable of degrading plasticisers only by co-metabolism increased with the relative inertness of the plasticiser as a microbial nutrient. For example 97% of bacteria grown on dipropyl phthalate (a plasticiser with high microbial resistance) showed co-metabolism, whereas only 46% of those grown on dibutyl sebacate (poor microbial resistance) required extraneous nutrients.

Control of biodeterioration

Materials in dry conditions are the least susceptible to biofilm formation and biodeterioration. Thus the best way to minimize biodeterioration is to maintain low humidity (Gaylarde & Morton, 1999). However, this is impossible in many situations, for

example outdoor applications of pPVC. Therefore, in order to control the microbial degradation of pPVC, biocides are often incorporated into the formulation of the plastic. An effective biocide should ideally meet a number of requirements. It should be active against a broad spectrum of microorganisms, be effective at low concentrations, be non-toxic to humans, be physically and chemically compatible with all the ingredients in the formulation, remain effective in the formulation throughout the useful life of the product, be low in cost, and environmentally acceptable (Lorenz, 1990). Generally it is impossible for any single biocide to satisfy all of these requirements. Biocides must therefore be carefully selected according to the intended application of the plastic. For example, pPVC for outdoor use must contain a biocide that is resistant to leaching by rainfall and to the effects of UV radiation and heat from sunlight. A broad range of industrial biocides are available, most of which can be classified within four groups according to their chemical structure and mode of antimicrobial activity (Table 3) (Gaylarde & Morton, 1999).

Table 3. Characteristics of biocide groups (Gaylarde & Morton, 1999)

Biocide Group	Characteristics	Examples
Oxidising Agents	Wide range of activity, can be low-cost	Halogens, ozone, organic peroxides
Protein denaturants and enzyme inactivators	Potential for specificity but often too ecotoxic	Aldehydes, phenolics, heavy metals, isothiazolones
Surfactive agents	Cleansing and dispersant activity, generally more environmentally acceptable	Quaternary ammonium or phosphonium compounds, household soaps
Sterol biosynthesis inhibitors	Fungicidal and algicidal only	Triazoles, imidazoles

Five commercial biocides commonly incorporated within pPVC were used in this study. These included N-(trichloromethylthio)phthalamide (NCMP), originally sold as an agricultural fungicide but shown to be suitable for incorporation into pPVC (Kaplan, 1970); 2-n-octyl-4-isothiazolin-3-one (OIT) originally patented as a combined antifungal and antibacterial agent for pPVC (Anon, 1967); n-butyl-1,2-benzisothiazolin-3-one (BBIT), an antifungal and antibacterial agent; 10,10, oxybisphenoxyarsine (OBPA), an arsenic-containing biocide originally patented as an antibacterial agent for PVC sheets and films (Lutz, 1973); and 2,3,5,6 tetrachloro-4(methyl sulphonyl) pyridine (TCMP), originally sold as a latex paint mildewcide (Trotz & Pitts, 1981) but subsequently found to be active against both fungi and gram positive bacteria (Wolf & Bobalek, 1967). All of

these biocides act at the surface of the pPVC and are thought to exert their activity through the denaturation of proteins and inactivation of enzymes. Isothiazolinone biocides, such as BBIT and OIT are known to target sulfhydryl groups within enzymes (Fuller *et al.*, 1985; Sondossi *et al.*, 1993). Currently OBPA dominates the market for biocides. Its future is uncertain however, as all biocides containing heavy metals are under threat from European legislation. Consequently there is continual competition to develop new biocides that could replace OBPA. Critical to the development of new biocides is their performance in a range of standard tests designed to compare biodeterioration in pPVC samples.

Test methods

A number of standard tests are employed to compare the susceptibility of different pPVC formulations to biodeterioration. These procedures are also used to compare the efficacy of biocides in controlling microbial attack. The principal test methods used are mineral salts agar, soil burial or humidity chamber tests (Cadmus, 1978).

Mineral salts agar tests

Mineral salts agar (MSA) tests involve covering of pPVC samples with a basal mineral salts agar seeded with a number of fungi or bacteria in a Petri dish (International Organisation for Standardization (ISO), 1978; American Society for Testing and Materials (ASTM) 1985a; ASTM 1985b). To test for the general susceptibility to microbial attack, no carbon source is added to the medium. If the organisms are to grow, they will therefore have to derive their nutrients from the test specimen itself. Growth will then appear on the test piece, not on the agar, indicating microbial susceptibility of the plastic (Cadmus, 1978). The test organisms in the ISO (1978) method include the fungi *Aspergillus niger*, *Penicillium funiculosum*, *Paecilomyces variotii*, *Trichoderma viride* and *Chaetomium globosum* and the bacterium *Pseudomonas aeruginosa*. In the ASTM methods *T. viride* and *C. globosum* are replaced by *Gliocladium virens* and *Aureobasidium pullulans*. For biocide tests, a carbon source is incorporated within the agar. The organisms grow rapidly on the medium, except in the area around the test specimen protected by the biocide. The level of protection conferred by a biocide is based on measurement of a zone of inhibition around the plastic (Bessems, 1988).

Soil burial tests

In soil burial methods strips of the test pPVC with or without incorporated biocides are buried about 2.5 cm below the surface of a rich soil bed and incubated for periods from 2 weeks to up to 32 months (Colin *et al.*, 1981; Seal & Pantke, 1988). This method is mainly used for pPVC formulations that come into contact with soil, for example pPVC used in building construction or cable insulation. Assessment of resistance to microorganisms is usually based on visual scoring of the extent of microbial growth, changes in mass, or changes in tensile strength caused by loss of plasticisers from the pPVC (Seal & Pantke, 1988). For example, Hueck (1974) measured the tensile strength of pPVC samples during soil burial. The results showed a progressive increase in tensile strength of up to 65% after 16 weeks of soil as the removal of plasticiser converted the soft polymer into tough and rigid PVC. In addition to measuring changes in the physical properties, chemical changes in the pPVC during degradation may also be monitored by Fourier transform infrared (FTIR) spectroscopy (Santoro & Koestler, 1991).

Humidity chamber tests

Humidity chamber tests involve spray inoculation of samples with a suspension of fungal spores and incubation for 30, 60 or 90 days in a humidity chamber maintained typically at 25°C and with a relative humidity of 98-100% (Cadmus *et al.*, 1978). Susceptibility to microbial attack is usually based on visual assessment of surface growth.

However, all of these tests have been criticised and may not provide an accurate estimation of the efficacy of biocides in controlling biodeterioration of the pPVC for a number of reasons. Firstly, zones of inhibition around plastic samples may be misleading because the water solubility of the biocide will determine its diffusion out of the pPVC and into the agar (Lorenz, 1990). Thus the zone of inhibition may be more indicative of the water solubility of the biocide, which determines its diffusion out of the pPVC and into the agar, rather than a measure of its protective potential.

Secondly these tests are normally based on a subjective assessment of visible growth, and may therefore vary depending on the investigator. Typically growth is rated from 0 to 3 depending on the extent of overgrowth of the test substrate. The tests therefore provide no information on whether microorganisms on the surface of the material are viable or have been killed by the activity of the biocide. It has been suggested that visual assessment techniques could be improved considerably by recording the presence or

absence of microbial growth in randomly selected fields of view using light microscopy (Santoro & Koestler, 1991).

Thirdly, the results of the test depend largely on the inoculum used. However, type-strains recommended for use in test procedures were often first isolated 50 – 60 years ago (Kelley & Yaghmaie, 1988). The characteristics of these organisms, in particular their ability to degrade plasticisers, may well have been altered through long-term preservation. In addition, many of the isolates used in standard tests were not isolated from plastics but have come to be adapted from other areas, for example textile testing. Therefore these microorganisms may not represent the population of microorganisms that cause biodeterioration of pPVC in the natural or service environments.

Therefore there is a need to determine which organisms are important in causing biodeterioration of pPVC *in-situ,* and to develop accurate and quantitative tests to monitor the susceptibility of these organisms to biocides incorporated in pPVC.

Screening of organisms for plasticiser degradation.

Several methods for the determination of the microbial degradation of plasticisers have been proposed. The simplest and most widely used method for identifying deteriogenic organisms is the homogenised plasticiser agar technique. This method consists of homogenising the plasticiser to be tested within a basal agar medium so that the plasticiser forms an emulsion that is translucent or opaque. Organisms capable of degrading the plasticiser will form a clear zone or halo around the area of growth when incubated on plates of this emulsified medium, thought to be caused by the production of extracellular esterases (Berk *et al.,* 1957; Klausmeier & Jones, 1961). Klausmeier & Jones (1961) also assessed plasticiser degradation by measuring the increase of the cell weight of microorganisms growing in the presence of plasticiser. Burgess & Derby (1965) devised a respirometric technique and a weight-loss method for comparing the degradation of plasticisers by *Pseudomonas aeruginosa.*

All of these methods also have disadvantages however. The plasticiser agar method is a qualitative test only and it is possible that some organisms may attack the plasticiser but give no clearing on the emulsion agar (Cavett & Woodrow, 1968). The cell weight method, although quantitative, involves the separation and accurate weighing of cell material which is technically difficult. The respirometric technique of Burgess & Derby (1965) gave only semi-quantitative data because fungal inocula were applied by an

atomizer which is difficult to standardise (Cavett & Woodrow, 1968). Weight loss experiments are also generally hindered by long incubation periods which can extend over weeks or months. Most of these problems can be overcome with the use of more advanced analytical techniques. For example, Sugatt *et al.* (1984) used gas chromatography to monitor the biodegradation of 14 commercial phthalate esters. In addition, infrared (IR) spectroscopy has been used to monitor degradation of plasticisers within pPVC (Hill, 1982; Gardette & Lemaire, 1991).

In-situ microbial colonization of pPVC

Although no detailed quantitative studies have been published, fungi are reported to be the predominant organisms that colonize pPVC surfaces in structural and outdoor applications of pPVC (Bessems, 1988; Hamilton, 1983). This may be due to the ability of fungi to withstand harsh environmental conditions, for example desiccation, UV irradiation and high temperatures. It has also been suggested that fungi are the principal deteriogenic organisms because they are more readily able to develop the enzymatic systems necessary to cleave the ester bonds of plasticisers (Lorenz, 1990). However, there are no studies in the literature that compare the relative roles of fungi and bacteria in the biodeterioration of pPVC *in-situ*.

Several studies have examined the fungal defacement of pPVC under tropical exposure conditions. For example, species of *Cladosporium*, *Alternaria*, *Curvularia*, *Nigrospora*, *Aureobasidium*, *Aspergillus*, *Trichoderma*, *Penicillium*, and *Paecilomyces* were isolated from pPVC exposed for 12 months in Puerto Rico (Hamilton, 1983). Upsher & Roseblade (1984) found species of *Fusarium*, *Aureobasidium*, *Cladosporium* and *Rhinocladiella* to be the dominant on pPVC pieces exposed in Queensland, Australia. However, in both studies fungal growth was evaluated using subjective, visual assessment of defacement of the pPVC. Neither examined the role of bacteria in the colonization process. In addition, most fungi were only identified to the genus level using basic morphological techniques.

Identification of Fungi

In fungi, techniques most commonly used for identification rely on the appearance of cellular or colony morphology. These tests are often laborious and may also be ambiguous because of strain variability. Morphological studies also have the major disadvantage that they may be influenced by the environmental conditions in which the

organism is grown. Because of these difficulties, molecular comparisons are used increasingly for fungal identification. Molecular comparisons that may allow differentiation between species include those of random amplified polymorphic DNA (RAPD) (Hadrys *et al.*, 1992), restriction fragment length polymorphisms (RFLP) (Bruns *et al.*, 1991) or rDNA sequences (Kurtzman & Robnett, 1998). Of these, rDNA sequencing is most frequently used because strain comparisons are more easily made (Kurtzman & Robnett, 1998).

The ribosomal structures present in all cells are an integral part of the protein translational system, and thus the genes encoding these structures are present in all organisms. In fungi the major structural rRNAs are the 18S, 5.8S and 28S subunits. The genes encoding these rRNAs are highly conserved (Bruns *et al.*, 1991). However, this conservation is not uniform throughout each gene and several variable regions are interspersed with the highly conserved domains. Internal transcribed spacer (ITS) regions that separate the rRNA genes also exhibit variability. The variable regions are useful for the identification of species (Kurtzman & Robnett, 1998), and for the study of phylogenetic relations between organisms (Bruns *et al.*, 1991).

Universal primers have been designed from conserved regions of rDNA that allow polymerase chain reaction (PCR) amplification of the variable ITS, 5.8S, 18S or 28S rDNA regions (Fell, 1993; White *et al.*, 1990). Amplified PCR products may then be sequenced for comparison between species. For example, Peterson & Kurtzman (1991) sequenced the variable D2 domain near the 5' end of large subunit (28S) rRNA from closely related yeasts in the genera *Isatchenkia*, *Pichia* and *Saccharomyces*. Strains of the same species generally had greater than 99% similarity in nucleotide sequences, whereas different species had lower similarity, thus providing a method for differentiation of the species. In bacteria, 16S subunit rDNA sequences have been analysed extensively for phylogenetic studies and identification purposes. Among bacteria, if two species have 100% identical 16S rDNA sequences, then the strains usually have high genomic DNA similarity and are thus the same species. If two organisms show little similarity in the 16S rDNA gene, then the organisms are distinct species with low genomic DNA homology. However, if two species differ only in a few nucleotides, the variation in genomic DNA can range from 23% to almost 100% (Fox *et al.*, 1992). Thus the similarity of rDNA sequences in isolation is not a guarantee of species identity and must be supported by examination of other factors such as morphological characteristics.

In this study, fungal isolates were identified firstly by PCR amplification and partial sequencing of the ITS regions and 5.8S rDNA, or of the 28S rDNA (Fig. 1). The sequences obtained were compared with known sequences in the EMBL fungal DNA database (EMFUN). The rDNA identification of each isolate was then confirmed by comparison with published descriptions of colony and conidial morphology.

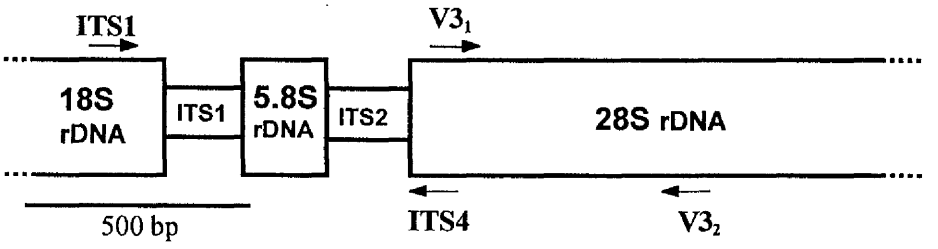


Fig. 1. rDNA and internal transcribed spacer (ITS) regions used in this study for the identification of fungi isolated from pPVC. ITS and 5.8S rDNA regions were amplified using universal fungal primers ITS1 and ITS 4 (White et al., 1990); 28S rDNA was amplified using primers $V3_1$ and $V3_2$ (Fell, 1993).

Airborne microorganisms

Microbial colonization of pPVC is probably influenced by the composition of the air microflora. The composition of the air microflora is determined locally by the source of microorganisms and by environmental parameters such as air currents, temperature and humidity (Lynch & Poole, 1979). Increased concentrations of some fungal spores (e.g. *Nigrospora* and *Cladosporium spp.*) released from plant surfaces have been associated with high temperatures and low relative humidity (Cammack, 1955). These environmental parameters may also influence the survival of airborne microorganisms and their ability to colonize surfaces after deposition. In general, fungal spores are resistant to the environmental stresses encountered during transport through the air. Bacteria are more susceptible, although bacterial endospores are well protected (e.g. *Bacillus* spp.). Yang *et al.*, (1993) found that *Cladosporium, Penicillium and Aspergillus* spp, basidiomycetes and *Alternaria* spp. were the five most predominant fungal taxa found in both indoor and outdoor air samples in the U.S. These results were in agreement with those reported by Strachan *et al.*, (1990) from British air, except that *Aureobasidium* spp. were also among the predominant organisms recovered.

In summary, nothing is known of the temporal colonization sequence that occurs on pPVC *in-situ*. In addition, the relative importance of colonizing organisms in the

biodeterioration of pPVC is poorly understood. This project therefore aimed to investigate the colonization process that occurs on pPVC *in-situ* and to identify to species level the predominant colonizing organisms using rDNA sequencing. A further aim was to examine the relationship between the observed colonization sequence and the ability of microorganisms to cause biodeterioration of pPVC in laboratory tests.

MICROBIAL ADHESION

Microbial adhesion is the first event that occurs during the colonization of a solid substratum (van Loosdrecht *et al.,* 1990). Understanding the mechanism of initial adhesion of microorganisms to plasticised PVC may therefore help in reducing or preventing colonization and biodeterioration processes.

In order to investigate the adhesion of microorganisms to surfaces it is necessary to investigate the physicochemical characteristics of the two surfaces and the interactions that occur between them (Oliveira, 1992). This study therefore aimed to investigate the physico-chemical factors influencing adhesion of *Aureobasidium pullulans* to pPVC because it has previously been found to be the predominant organism causing defacement of pPVC *in-situ* (Hamilton, 1983; Upsher and Roseblade, 1984)

In the case of pPVC exposed to the air *in-situ,* microorganisms that cause biodeterioration of the pPVC are deposited onto the substratum from the atmosphere. This process is influenced by environmental parameters such as air currents and rainfall (Lynch & Poole, 1979). Surfaces exposed to the atmosphere are subject to continual wetting/drying cycles. However, it is not known whether attachment occurs principally at the solid/air interface, or at the solid/liquid interface during outdoor exposure of materials. To date, there appears to be no studies of the interactions that occur between microorganisms and the surface at the solid/air interface. This lack of data and techniques makes it difficult to investigate the influence of the properties of the solid/air interface on adhesion.

In contrast to the limited amount of information concerning adhesion of microorganisms to the solid/air interface, attachment at the air/liquid interface has been researched extensively and the parameters influencing this process are well characterised (Wit & Busscher, 1998). Therefore in this study the mechanism of initial adhesion of *A. pullulans* to pPVC was investigated at the solid/liquid interface.

Theory of adhesion

Microbial adhesion to a surface within a liquid generally occurs as a three stage process involving firstly, transport to the surface, secondly, reversible adhesion and thirdly, irreversible adhesion as described by van Loosdrecht *et al.*, (1990).

Transport of microorganisms to the surface

Microorganisms in a liquid can reach a surface by several different mechanisms. The first is diffusive transport, where cells exhibit Brownian motion that can be observed under the microscope (van Loosdrecht *et al.*, 1990). This movement accounts for slow, random contacts of small bacteria with surfaces but is probably less relevant for larger fungal spores or yeast cells. The second mechanism is gravitational settling. This is especially relevant for the larger fungal propagules which may rapidly settle to a surface from suspension under quiescent conditions. Thirdly, cells may be transported by convective liquid flow, which can occur much faster than diffusive transport (Characklis, 1981). Convective forces have been demonstrated to be much more effective at transporting cells to the surface than Brownian motion (Rijnaarts *et al.*, 1993). Lastly, motile organisms may encounter a surface either by chance under their own motility, or by a chemotactic response towards concentration gradients established near the solid-liquid interface (van Loosdrecht *et al.*, 1990).

Initial (reversible) adhesion

The next step in the process is initial adhesion, which is mainly a physico-chemical interaction between the microorganism and the substratum. During initial adhesion, microorganisms in suspension may be considered as colloidal particles that obey physico-chemical forces such as those described by the Derjaguin-Landau and Verwey-Overbeek (DLVO) theory (Busscher & Weerkamp, 1987). The DLVO theory describes the change in Gibbs energy as a function of the distance between a particle and the substratum. The total Gibbs energy is the sum of the van der Waals forces and the electrostatic forces of interaction between the two bodies. At large separation distances of greater than 50 nm, only van der Waals forces operate, which are usually attractive (Busscher & Weerkamp, 1987). At separation distances between 10 and 20 nm, additional electrostatic forces become active. In nature most microorganisms and surfaces are negatively charged and therefore the electrostatic forces between them are repulsive. Thus a stage is reached where the repulsive electrostatic interactions balance the attractive van der Waals forces. Closer approach of the cell to the surface is thus prevented. The size of this energy barrier and the distance of the secondary minimum from the surface depend on the thickness of the double layer, which, in turn, is dependant on the valency and concentration of the electrolyte in the surrounding medium (van Loosdrecht *et al.*, 1990). The cell may therefore be held at this point known as the secondary minimum

(Fig. 2). At the secondary minimum the cell is said to be reversibly bound and may continue to exhibit Brownian motion or be removed from the surface by mild shear forces (van Loosdrecht *et al.*, 1990).

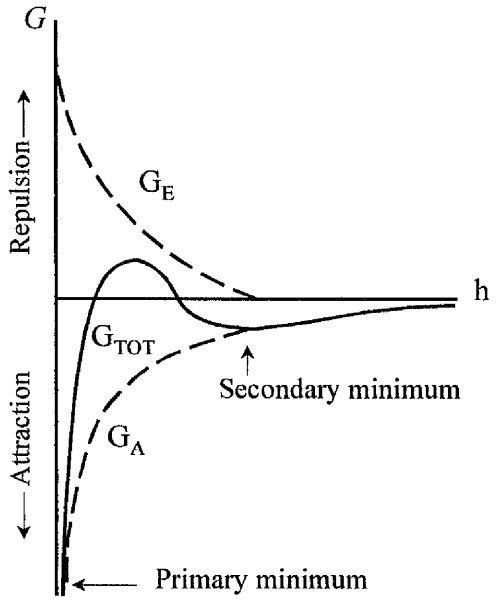


Fig. 2. Gibbs free energy of interaction (G) versus separation (h) for a charged particle approaching a surface of the same sign, at intermediate ionic strength. G_E represents the electrostatic interactions; G_A, van der Waals interactions and G_{TOT}, sum total of electrostatic and van der Waals interactions adapted (from Rutter & Vincent, 1980).

Irreversible Adhesion

Once the cell is positioned at the secondary minimum, this energy barrier may be overcome by bridging polymers produced by the cell, such as fimbriae or extracellular polymers (see Cell surface structures, p34). Because of their extremely small radii, such molecular surface structures may contact directly with the substratum through the energy barrier (Marshall, 1985). The hydrophobicity of the substratum may at this stage exclude the film of water between contacting areas, allowing the cell and the substratum to interact directly by short range hydrophobic and specific interactions (Busscher & Weerkamp, 1987). At this stage the cell is positioned within the primary minimum (Fig. 2) and is said to be irreversibly bound. It no longer exhibits brownian motion, and usually cannot be removed from the surface by simple washing procedures (Oliveira, 1992).

Co-operative adhesion

Adhesion of cells to a substratum without interaction with one another would result in adhesion in direct proportion to cell density. This is described by the equation B = KU (Henry's law) where B = number of bound cells, K = constant and U = number of unbound cells (Doyle, 1991). In the binding isotherm plot (Fig. 3), this would give the straight line relationship (a). However, this is rarely obeyed at all cell densities as saturation of the substratum binding sites may occur as in (b). Alternatively the binding of one cell may change the probability of other cells binding as in (c) with the adhesion of one cell to the surface making adhesion of other cells more energetically favourable. This is known as positive co-operativity (Doyle, 1991). This may be due to the binding of a cell to the surface causing configurational changes in the substratum creating new binding sites for other cells as suggested by Nesbitt *et al.*, (1982) to describe the positive co-operativity in the adhesion of *Streptococcus sanguis* to saliva-coated hydroxyapatite beads. Alternatively, negative co-operativity may occur, the occupancy of binding sites by cells reducing the probability of binding sites being filled. This may occur due to electrostatic repulsion (Klotz, 1985) or due to conformational changes in receptor sites (Doyle, 1991).

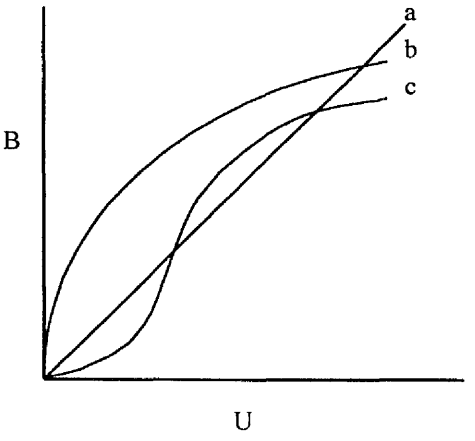


Fig. 3. Idealised binding isotherm plots of adhesion data where B = number of bound cells and U = unbound cells (Doyle, 1991). See text for description of plots a, b and c.

Factors influencing adhesion

Microbial adhesion to surfaces is a complex process that is influenced by many factors including the physico-chemical properties of the microorganism and the substratum. The principal physico-chemical factors that influence microbial adhesion are described below.

Properties of the microorganism.

Cell surface hydrophobicity

Hydrophobicity describes the level of interaction or bonding that occurs between a surface and water. Surfaces with low hydrophobicity are readily able to interact with water molecules through van der Waals forces, hydrogen bonds and polar interactions (Fletcher, 1991). Surfaces with high hydrophobicity do not readily interact with water. As a result, the water becomes less structured close to the surface in terms of intermolecular hydrogen bonding between the water molecules. The term 'surface energy' is often used in conjunction with hydrophobicity. Surface energy describes the potential for molecules at a surface to enter into a range of interactions, whereas hydrophobicity is determined by these interactions with water only. In general, hydrophobicity decreases with increasing surface free energy (Fletcher, 1991).

Several methods are available to determine the hydrophobic or hydrophilic nature of the cell surface. These include sessile drop water contact angle measurements (Busscher *et al.*, 1984), evaluation of the ability of cells to attach to hydrophobic hexadecane or polystyrene (Rosenberg *et al.*, 1980; Goldberg *et al.*, 1990), partitioning of cells in an aqueous two-phase system (van Loosdrecht *et al.*, 1987b), salt aggregation tests (Lindahl *et al.*, 1981), or hydrophobic interaction chromatography (Mozes & Rouxhet, 1987).

In bacteria, cell surface hydrophobicity varies according to the bacterial species and is influenced by the growth medium and phase of growth of the organism (Krekeler *et al.*, 1989). Generally, cells with hydrophobic surfaces adhere in greater numbers to hydrophobic material surfaces, while more hydrophilic cells attach preferably to hydrophilic surfaces. For example, Hogt *et al.*, (1986) found that a hydrophobic strain of *Staphylococcus epidermidis* showed significantly higher levels of adhesion to hydrophobic fluorinated poly (ethylenepropylene) than did a relatively hydrophilic strain of *S. saphrolyticus*. Williams & Fletcher, (1996) found that mutants of *P. fluorescens* with increased hydrophobicity showed increased attachment to hydrophobic polystyrene. Cell surface hydrophobicity has also been shown to be important in the adhesion of fungi to surfaces. The yeast *C. albicans* showed increased adhesion to hydrophobic plastics with increased hydrophobicity of the cell surface (Miyake *et al.*, 1986; Panagoda *et al.*, 1998).

Electrostatic properties of the cell surface

Cell surface charge may also influence microbial adhesion. As with cell surface hydrophobicity, cell surface charge varies according to the organism, growth medium, phase of growth and cell surface structure (Dankert *et al.*, 1986). The surface charge of a cell is usually characterized by its electrokinetic potential (or zeta potential), or electrophoretic mobility (van Loosdrecht *et al.*, 1987a; van der Mei *et al.*, 1993). The zeta potential of a cell is the potential generated at the plane of shear when two phases move relative to one another on application of an electric field (James, 1991). Microbial cells in aqueous suspensions are generally negatively charged and a high surface charge is usually accompanied by a hydrophilic characteristic of the organism (An & Friedman, 1997). The adhesion of several hydrophilic organisms such as *Escherichia coli* (Gilbert *et al.*, 1991), *Saccharomyces cerevisiae* and *Acetobacter aceti* (Mozes *et al.*, 1987) has been found to be influenced by electrostatic interactions. However, many studies have also found that adhesion of bacteria to a range of surfaces is not affected by the relative surface charge of the bacteria (Abbott *et al.*, 1983; Hogt *et al.*, 1985). Therefore the role of cell surface charge in adhesion varies according to the organism and substratum in question.

Cell surface structures

The topographical properties of the cell surface may also affect adhesion. In bacteria, extracellular polymers, fimbriae, fibrils and flagella may facilitate adhesion directly by bridging of the organism to the surface (Marshall, 1985). Many fungi also have surface structures which may aid initial attachment. For example, a number of fungi have spores with mucilaginous sheaths e.g. *Pleospora spp.* (Hyde *et al.*, 1986) and *Halosphaeria salina* (Zainal & Jones, 1984). *A. pullulans* hyphae and blastospores often produce an EPS capsule (Andrews *et al.*, 1994) which may aid in their adhesion to pPVC. Fimbriae have been reported on the surface of a number of spores, e.g. sporidium of *Ustilgo violaceae* (Day & Poon, 1975), and ascospores of *Nereiospora* spp. have tufts of hair-like polar and equatorial appendages which appear to anchor spores to surfaces (Jones *et al.*, 1983). In addition to their bridging role, cell surface structures in fungi and bacteria probably also contribute to the overall cell surface characteristics such as hydrophobicity and cell surface charge (An & Friedman, 1997).

Properties of the substratum

Substratum hydrophobicity

In general, increased substratum hydrophobicity is associated with increased adhesion in both bacteria (Fletcher & Loeb, 1979; Miller & Ahearn 1987) and fungi (Klotz *et al.*, 1985; Doss *et al.*, 1993). For example, Fletcher & Loeb (1979) showed a linear relationship between surface hydrophobicity and adhesion of a marine pseudomonad. However, this is not exclusively the case as increased adhesion of *Staphylococcus sanguis* has been shown to occur to hydrophilic rather than hydrophobic substrata (Busscher *et al.*, 1990). In addition, Denyer *et al.*, (1993) showed that cells of *Staphylococcus epidermidis* have optimal ranges of hydrophobicity within which they show maximum adhesion. Thus the effect of substratum hydrophobicity may vary depending on the organism and substratum studied.

Substratum hydrophobicity is normally measured using the water contact angle. This is the angle formed by the tangent to the point of contact at the solid/water/air interface. Methods used to determine water contact angles on solid surfaces include sessile drop and Wilhelmy plate techniques. The sessile drop technique measures the angle of a water droplet placed on the substratum using a syringe. Measurement of the angle is achieved either by telescopic (Fletcher & Marshall, 1982) or laser beam goniometry (Lander *et al.*, 1993). The Wilhelmy plate technique measures contact angles using a dynamic contact angle analyser (DCA). The DCA measures the force required to dip the test surface into a liquid of known surface tension using an electrobalance. The force is measured as the test surface advances or recedes from the liquid. The contact angle is then measured from the equation $\cos\theta = F/p.y$ where F is the force measured by the electrobalance (dynes), p is the perimeter of the sample (mm) and y is the surface tension of the wetting liquid (dynes cm^{-1}) (Domingue, 1990). In this study the Wilhelmy plate technique was used to measure surface hydrophobicity because it has been shown to be a more accurate and reproducible technique than sessile drop methods due to the large surface area that can be sampled (Lander *et al.*, 1993).

Electrostatic properties of the substratum

The electrostatic properties of the substratum may affect adhesion particularly on hydrophilic surfaces. However, there are relatively few studies of role of surface charge in adhesion. Fletcher & Loeb (1979), found that adhesion was low on negatively charged hydrophilic surfaces, such as glass, but high on neutral (germanium) or positively

charged (platinum) hydrophilic surfaces. However, Harkes *et al.*, (1992) found no correlation between adhesion and substratum charge for *E. coli* cells. Thus the role of electrostatic properties of the substratum probably varies according to the combination of organism and substratum in question.

The electrostatic properties of a surface may be measured by the potential at the surface of shear as the surface moves relative to the surrounding fluid. This is known as the zeta potential (James, 1991). The zeta potential of a flat planar surface may be determined by using the streaming potential method. This technique measures the potential generated when a fluid is moved with respect to a charged surface (van Wagenen & Andrade, 1980).

Surface Roughness

In addition to the hydrophobic and electrostatic properties of the substratum, surface roughness can play an important role in microbial adhesion. Increased surface roughness has been shown to increase adhesion of *P. fluorescens* (Mueller *et al.*, 1992), although this increase was strain specific. McAllister *et al.*, (1993) also found that irregularities of polymeric surfaces can promote bacterial adhesion and biofilm formation. Surface roughness may increase adhesion by providing increased surface area for adhesion or protection from shear forces (Quirynen & Bollen, 1995). Surface roughness may be measured using a profilometer which uses a stylus to trace over the surface, producing a surface profile (Sander, 1991).

Properties of the wetting fluid.

Many studies have shown that the concentration of electrolyte within the wetting medium is an important factor influencing adhesion. For example, increasing ionic strengths causes increased adhesion in *Candida albicans* (Jones & O'shea 1994). Similarly, adhesion of *P. aeruginosa* to stainless steel at low ionic strengths (up to 10 mM), was shown to increase with increasing electrolyte concentration (Stanley, 1983). Increasing the valency of ions within the wetting liquid may also result in increased adhesion (Marshall *et al.*, 1971). The influence of the concentration and valency of the electrolyte on adhesion is attributed to the thickness of the double-layer of counter ions between the microorganism and the substratum. With increasing ionic strength or valency, this double layer is compressed, allowing closer approach of the cell to the substratum before it experiences electrostatic repulsion (van Loosdrecht *et al.*, 1989).

However, this is not always the case as McEldowney and Fletcher (1986) found no correlation between adhesion of *Pseudomonas fluorescens* and electrolyte concentration or valency. Therefore the influence of the electrolyte may vary depending on the microorganism and the substratum in question.

In addition to the concentration of the electrolyte, the pH of the wetting liquid can also influence adhesion. The optimum pH for adhesion generally depends upon the isoelectric point of the microbial cell (Daniels, 1980). The isoelectric point is the pH at which the net surface charge due to ionization of acidic or basic groups on the cell surface is zero. If the pH of a suspension containing microbial cells is above the isoelectric point of carboxyl groups on the cell surface, then these groups dissociate to produce hydrogen ions. The cell then exhibits a net negative charge. At pH values below the isoelectric point, carboxyl groups on the cell surface are undissociated and additional protonation of the cell surface occurs. The cell therefore takes on a net positive charge (Daniels, 1980). Thus the pH of the wetting liquid may influence adhesion by affecting the surface charge of the microorganism. For example, adhesion of *Bacillus cereus* spores to glass surfaces was found to be greatly reduced at pH values above the isoelectric point of the spores (pH 3) (Husmark & Rönner, 1990). This reduction in adhesion was thought to be due to electrostatic repulsion between the spore and glass surfaces which are both negatively charged at high pH. In general, the strongest adhesion of microorganisms to surfaces occurrs at pH 3 – 6 (for a review, see Daniels, 1980).

Adhesion may therefore be influenced by a range of properties of the microbial cell, substratum, and wetting liquid. However, there appears to be no studies of the mechanism of adhesion of deteriogenic fungi during the colonization of pPVC. This study therefore aimed to investigate the physico-chemical nature of adhesion of *A. pullulans* to pPVC.

GREEN FLUORESCENT PROTEIN

In order to study colonization of pPVC by *A. pullulans* and to investigate the susceptibility of this organism to biocides incorporated in pPVC, it was necessary to develop techniques to monitor growth and viability of living *A. pullulans* colonies on the pPVC substratum *in-situ.* The ideal reporter system for these studies would allow easy imaging and quantitation of *A. pullulans* in real-time on the pPVC substratum and avoid the need for chemical substrates or dyes. The green fluorescent protein (GFP) from the jellyfish *Aequoria victoria* is intrinsically fluorescent, requiring no substrates or co-factors (Chalfie, 1995). The application of GFP as a reporter for *A. pullulans* growth and viability on pPVC was therefore investigated.

Green fluorescent protein (GFP) was originally cloned from the bioluminescent jellyfish *Aequoria victoria* by Prasher *et al.*, (1992). Chalfie *et al.*, (1994) subsequently demonstrated that expression of the gene in both prokaryotic and eukaryotic organisms causes green fluorescence, and that the gene contains all the information necessary for the post-translational synthesis of the GFP chromaphore. Since these studies GFP has been expressed in a wide range of heterologous systems and has become established as a powerful reporter for living cells and for gene expression in a wide variety of experimental systems (Prasher, 1995; Kain, 1999).

GFP and bioluminescence

GFPs are involved in bioluminescence of a variety of coelenterates, including hydrozoa such as *Aequoria*, *Obelia* and *Phialadium*, and anthozoa such as *Renilla*. GFPs occur as partners with chemiluminescent proteins (luciferins) and control the colour of the emission *in-vivo*. Bioluminescent reactions require three principal components: a luciferin, a luciferase and molecular oxygen. A luciferase is an enzyme that catalyses the oxidation of a substrate, luciferin, that subsequently emits light. The coelenterate luciferin is called coelenterazine (Mager & Tu, 1995). GFPs emit green light having accepted energy from luciferins or photoproteins, depending on the species. In *Aequoria* coelenterazine is bound to the photoprotein aequorin. Aequorin responds directly to Ca^{2+} by oxidizing the pre-bound coelenterazine (luciferin), and the energy released stimulates GFP to emit green light in-vivo (Morin & Hastings, 1971) (Fig. 4). In the absence of GFP, aequorin will release blue light *in-vitro.* This process occurs in specialized

photogenic cells located at the base of the jellyfish umbrella and is thought to provide either a communication function or a defence mechanism to the organism.

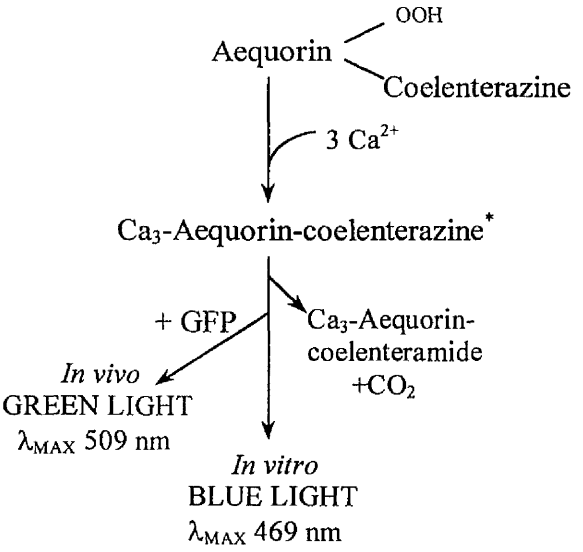


Fig. 4. The bioluminescent pathway in *Aequoria*. The luciferin involved is coelenterazine and the photoprotein aequorin responds directly to Ca^{2+} by oxidising the bound coelenterazine. The energy released stimulates GFP to emit green light *in-vivo* (from Prasher, 1995).

Structure of GFP

GFP is a 238 amino acid protein which is intrinsically fluorescent, encoding the light-emitting chromaphore within its primary amino-acid sequence. The highly fluorescent chromaphore is formed from amino acids 65-67 within the protein which are Ser-Tyr-Gly respectively. These three residues are cyclized and oxidized post-translationally to form the p-hydroxybenzylideneimidazolinone chromaphore (Prasher *et al.*, 1992). The proposed structure of the chromaphore is shown in Fig. 5. The chromaphore is positioned almost perfectly in the centre of a protective 11-stranded β-barrel (Tsien, 1998).

GFP fluorescence

GFP absorbs blue light with maxima at 395 nm and 470 nm and emits green light with a maximum of 507 nm. Because of the protective environment of the GFP chromaphore, the resultant GFP protein is highly stable, remaining fluorescent up to 65°C, 1% sodium dodecyl sulphate, and resisting most proteases for many hours (Cubitt *et al.*, 1995). Once GFP is synthesised it can be detected by irradiation with near UV or blue light and is not limited by the availability of substrate (Chalfie *et al.*, 1994). Because fluorescence

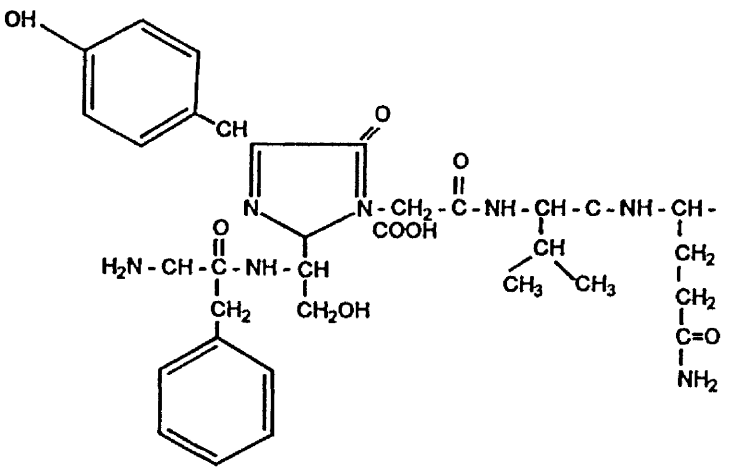


Fig. 5. Proposed structure of the GFP chromaphore peptide from *Aequoria victoria* (from Cody *et al.*, 1993).

requires no cofactors, the GFP signal can be recorded in living cells with no disruption of cellular integrity. Fluorescence can be measured at the level of a cell population with luminometers or at the single cell level with fluorescence microscopy or fluorescence activated cell sorting (FACS) (Cormack *et al.*, 1996).

pH sensitivity of GFP

Early studies on wild-type GFP noted the pH sensitivity of fluorescence (Ward *et al.*, 1982; Ward & Bokman, 1982). Fluorescence in wild type GFP is lost at acidic pH values, with 50% reduction in fluorescence occurring at pH 4.5 (Tsien, 1998). The underlying mechanism of pH-dependant changes in fluorescence is not fully understood but is thought to involve altered protonation of the chromaphore (Robey *et al.*, 1998). These changes are both rapid and readily reversible for wild-type GFP *in-vitro*, even at extremely acidic pHs (Ward *et al.*, 1982). The sensitivity of some GFPs to pH has both advantages and disadvantages. Such GFPs may not be useful for studies of acidic organelles such as lysosomes and golgi apparatus. However, the pH sensitivity of GFP has recently been useful in the measurement of cytosolic and organellar pH (Llopis *et al.*, 1998; Robey *et al.*, 1998).

GFP variants

The native version of the *gfp* gene is often poorly expressed in heterologous systems and requires modification. Most mutations in GFP result in a partial or complete loss of

fluorescence without significant change in the relative absorption or emission peaks. These mutations probably cause misfolding of the protein, failure of chromaphore formation, or quenching of the fluorescence by insufficient shielding. However, a number of 'red-shifted' variants of wild-type GFP have been generated by several investigators, most of which contain one or more amino acid substitutions in the chromaphore region of the protein (Yang *et al.*, 1996; Kain *et al.*, 1995). The term red-shifted refers to the position of the major fluorescence excitation peak, which is shifted for each of these variants towards the red, from 395 nm in wt GFP to 488-490 nm (Kain, 1999). The major excitation peak then encompasses the excitation wavelength of commonly used excitation filter sets (such as those used for fluorescein) and thus the resulting signal is much brighter relative to wt GFP. This property, combined with more efficient folding of the protein that occurs in these variants has resulted in increases in fluorescence intensity of up to 100-fold relative to the wild-type protein (Cormack *et al.*, 1996).

The two most commonly used red-shifted GFP variants are Ser65Thr (Heim *et al.*, 1995), which contains a Ser65 to Thr substitution in the chromaphore, and GFPmut1 or enhanced GFP (EGFP) (Cormack *et al.*, 1996), which contains the same Ser65Thr change plus a Phe64Leu mutation. The EGFP coding DNA sequence has also been modified with 190 silent base changes to contain codons that are preferentially found in highly expressed human proteins (Haas *et al.*, 1996.) These changes contribute to efficient expression in mammalian cells and subsequently very bright fluorescence signals. Advantages of EGFP applications include improved sensitivity of detection, faster chromaphore oxidation to form the fluorescent form of the protein and reduced rates of photobleaching (Kain, 1999).

Applications of GFP

GFP may be used as a tag or as an active indicator of physiological events within cells. As a tag, GFP has been used extensively in a wide range of prokaryotic and eukaryotic systems to assess gene expression, as a cellular label to monitor host-parasite interactions and to monitor protein localization (for a review, see Tsien, 1998). As an indicator, GFP fluorescence is influenced post-translationally by its chemical environment. For example, the pH-sensitivity of GFP has recently been exploited to measure intracellular (Kneen *et al.*, 1998; Robey *et al.*, 1998; Llopis *et al.*, 1998) and organellar (Llopis *et al.*, 1998) pH, and GFP-based systems have been developed to monitor intracellular calcium

(Miyawaki *et al.*, 1997), microviscosity (Swaminathan *et al.*, 1997) and protease activity (Heim & Tsien, 1996).

GFP expression in fungi.

A combination of highly fluorescent GFP mutations (see GFP variants, p40) and optimized codon usage has recently allowed efficient GFP expression in a variety of fungi including *C. albicans* (Cormack *et al.*, 1997), *Aspergillus spp.* (Fernández-Ábalos *et al.*, 1998; Gordon *et al.*, 2000), *U. maydis* (Spellig *et al.*, 1996) and *A. pullulans* (Vanden Wymelenberg *et al.*, 1997) (Fig. 5).

In *A. pullulans,* GFP expression was used as a cell marker and allowed quantification of the fungus on leaf surfaces. In this study, *A. pullulans* is of interest because it is reported to be the predominant organism causing defacement of pPVC in outdoor exposure trials (Hamilton, 1983; Upsher & Roseblade, 1984). GFP expression in *A. pullulans* may therefore provide a useful tool for monitoring colonization and growth of this fungus on pPVC in real-time.

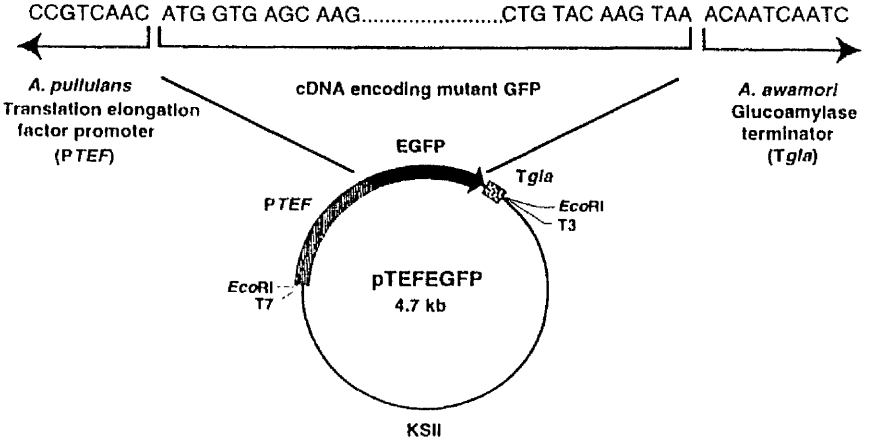


Fig. 6. The expression vector pTEFEGFP. A red shifted, engineered mutant GFP cDNA (pEGFP) is fused to the translation elongation factor (TEF) promoter from *A. pullulans* and the glucoamylase terminator from *Aspergillus awamori.* The entire expression cassette is ligated into pBluescript II KS(-) and the sequence of junctions was confirmed by dideoxy sequencing. Partial sequence of the relevant regions is shown (from Vanden Wymelenberg *et al.*, 1997).

Several recent findings have also led to the speculation that GFP may be a useful as a reporter for antimicrobial susceptibility in *A. pullulans*: i) GFP has been used as an accurate and sensitive indicator of intracellular pH (Kneen *et al.*, 1998; Robey *et al.*, 1998); ii) several studies have correlated intracellular pH with fungal cell viability (Imai & Ohno, 1995; Viegas *et al.*, 1999). It was therefore hypothesised that biocides effective against *A. pullulans* would result in loss of intracellular pH regulation and loss of GFP fluorescence. The potential use of GFP as a reporter to study colonization of pPVC by *A. pullulans,* and as an indicator of antimicrobial susceptibility, was investigated in this study.

OVERALL AIMS

The overall aims of this project were to investigate microbial colonization processes that occur on pPVC and to develop novel techniques to compare the efficacy of biocides in preventing microbial growth on the pPVC substratum. The following specific objectives were therefore identified:

Microbial colonization and biodeterioration of pPVC

- To examine the microbial colonization of pPVC *in-situ* by exposing pPVC to the atmosphere in a longitudinal experiment.
- To identify important colonizing organisms to species level using rDNA sequencing.
- To determine whether laboratory tests used to assess the ability of microorganisms to cause biodeterioration of pPVC are useful in predicting which organisms are important in the colonization of pPVC *in-situ*.

Adhesion of *Aureobasidium pullulans* to pPVC

- To develop an adhesion assay to quantify adhesion of *A. pullulans* to pPVC.
- To investigate the mechanism of adhesion of *A. pullulans* to pPVC by studying the physico-chemical factors, such as hydrophobic and electrostatic interactions, that influence adhesion to pPVC.

GFP as a novel indicator of antimicrobial susceptibility in *A. pullulans*

- To evaluate the green fluorescent protein (GFP) as a potential reporter molecule in *Aureobasidium pullulans* to allow non-invasive and real-time monitoring of fungal susceptibility to antimicrobial compounds.

- To compare the efficacy of a range of biocides commonly incorporated in pPVC against *A. pullulans* in liquid suspension.

GFP as a reporter to study growth *A. pullulans* on pPVC

- To evaluate GFP as a reporter for living *A. pullulans* cells in order to study growth and morphogenesis of *A. pullulans* on pPVC in real-time using GFP as a reporter for living cells.
- To investigate the effect of biocides incorporated within pPVC on the viability of attached *A. pullulans* cells using GFP as a reporter for cell viability.

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Chapter 2.

Fungal colonization and biodeterioration of plasticized polyvinyl chloride

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ABSTRACT

Significant substratum damage can occur when plasticized polyvinyl chloride (pPVC) is colonized by microorganisms. We investigated microbial colonization of pPVC in an *in-situ*, longitudinal study. Pieces of pPVC containing the plasticizers di-octyl phthalate (DOP) and di-octyl adipate (DOA) were exposed to the atmosphere for up to two years. Fungal and bacterial populations were quantified, and colonizing fungi were identified with rDNA sequence and morphological characters. *Aureobasidium pullulans* was the principal colonizing fungus, establishing on the pPVC between 25 and 40 weeks of exposure. A group of yeasts and yeast-like fungi, including *Rhodotorula aurantica* and *Kluyveromyces spp.*, established much later (after 80 weeks of exposure). Numerically, these organisms dominated *A. pullulans* after 95 weeks with a mean viable count and standard error of 1000 ± 200 yeast CFU cm^{-2}, compared to 390 ± 50 *A. pullulans* CFU cm^{-2}. No bacterial colonization was observed. We also used *in-vitro* tests to characterize the deteriogenic properties of fungi isolated from the pPVC. All strains of *A. pullulans* tested could grow with the intact pPVC formulation as the sole source of carbon, degrade the plasticizer DOA, produce extracellular esterase, and cause weight loss of the substratum during growth *in-vitro*. In contrast, several yeast isolates could not grow on pPVC or degrade DOA. These results suggest that microbial succession may occur during the colonization of pPVC and that *A. pullulans* is critical to the establishment of a microbial community on pPVC.

INTRODUCTION

Plasticized PVC (pPVC) is highly susceptible to microbial attack in many different environmental situations. The problem was first identified in U.S. Government reports of the deterioration of military equipment (Brown, 1945; Wellman & McCallan, 1945), and subsequent reports described defacement and deterioration of commercial pPVC products (Yeager, 1952; Girard & Koda 1959). Biodeterioration of pPVC is now known to occur in a wide range of industrial, commercial, and structural applications (Griffin & Uribe, 1984; Flemming, 1998; Gaylarde & Morton, 1999).

The susceptibility of pPVC results from the presence of plasticizers, commonly organic acid esters such as dioctyl phthalate (DOP) and dioctyl adipate (DOA), added to modify physical or mechanical properties of the polymer. Both bacteria (Booth *et al.*, 1968; Booth & Rob, 1968; Eaton & Ribbons, 1982) and fungi (Berk *et al.*, 1957; Roberts & Davidson, 1986) can degrade ester-based plasticizers. Loss of plasticizers from pPVC due to microbial degradation results in brittleness, shrinkage and ultimately failure of the pPVC in its intended application.

Microbial deterioration of pPVC has been studied extensively *in-vitro*. Many studies have examined the resistance of pPVC formulations incorporating biocides to colonization by test organisms (Santoro & Koestler, 1991; Seal & Pantke, 1988; Whitney, 1996). Other research has determined biodegradability by measuring changes in the physical properties of pPVC, such as changes in tensile strength (Yabannavar & Bartha, 1994), mass (Cavett & Woodrow, 1968), or electrical properties (Wasserbauer & Blahnik, 1975) during biodegradation. Several international standard test methods for microbiological susceptibility of plastics have been established (American Society for Testing and Materials (ASTM), 1985a; ASTM 1985b; International Organisation for Standardization (ISO), 1978).

Colonization processes occurring on pPVC in the environment have received comparatively little attention. Nothing is known about the temporal sequence of microbial colonization of pPVC *in-situ*. Existing studies have examined fungal defacement of pPVC in tropical or sub-tropical climates (Hamilton, 1983; Upsher & Roseblade, 1984). In both studies fungal growth was evaluated with a subjective, visual assessment of defacement of the pPVC. Neither study examined the role of bacteria in the colonization process. Further, unrecognized fungal growth was normally identified only to genus level with basic morphological techniques. Recently, rDNA sequencing

has been used as a rapid and reliable tool for the identification of fungi to species level (Guarro *et al.*, 1999). This technique has not been used to identify microorganisms that colonize and deteriorate pPVC in the environment.

We examined the microbial colonization of pPVC *in-situ* by exposing pPVC to the atmosphere in a longitudinal experiment. The principal objectives of this work were: (i) to investigate colonization processes, (ii) to identify important deteriogenic organisms to species level using rDNA sequencing, and (iii) to determine if there was a relationship between the microbial colonization sequence observed *in-situ* and the ability of microorganisms to cause biodeterioration of pPVC in laboratory tests.

MATERIALS AND METHODS

Culture media and maintenance

Fungi and yeasts enriched from pPVC exposed to the atmosphere were maintained on malt extract agar (MEA; Oxoid, Basingstoke, United Kingdom), a medium used widely for the detection, isolation and enumeration of fungi. Bacteria were maintained on R2A medium (Reasoner & Geldreich, 1985) (Difco, Detroit, MI), which contains low concentrations of organic nutrients and is used routinely for the enrichment of bacteria from oligotrophic environments. For long term storage at -80°C, fungal spores were harvested from agar plates and frozen in 20% (v/v) glycerol. The basal mineral salts medium (MSM) used for determining the deteriogenic properties of organisms contained the following (g l^{-1} distilled H_2O): K_2HPO_4, 7; KH_2PO_4, 3; $MgSO_4.7H_2O$, 0.1; and $(NH_4)_2SO_4$, 1. DOA agar, used for the isolation of organisms able to degrade the plasticizer DOA, contained MSM supplemented with 2 ml l^{-1} DOA and 15 g l^{-1} bacteriological agar (Oxoid). For preparation of DOA agar, medium including the DOA was autoclaved at 121°C for 15 min and allowed to cool to approximately 50°C. An emulsion of plasticizer was then created within the medium using a homogenizer at full power for 2 min (Ystral model D-7801, 260W, 25,000 rpm; Hemel Hempstead, United Kingdom). Plates were poured immediately after homogenization. DOA liquid medium was prepared exactly as DOA agar except without agar.

Plasticized PVC

Sheets of pPVC, 0.5 mm thick, were formulated that contained the following components (parts per hundred resin): EP 6779 PVC resin, (European Vinyls Corporation Ltd., Runcorne, United Kingdom), 75; Vinnolit C65V PVC resin (Vinnolit, Cologne, Germany), 25; Dioctyl phthalate plasticizer (Exxon Chemicals, Southampton, United Kingdom), 25; dioctyl adipate plasticizer (Exxon Chemicals), 25; Lankromark LN138 calcium/zinc stabilizer, (Akcros Chemicals, Burnley, United Kingdom), 2; Lankroflex ED63 epoxidized oleate ester (Akcros Chemicals), 3; and titanium dioxide pigment (Tioxide Europe, Grimsby, United Kingdom), 10. Individual pPVC pieces were 4.2 × 7 cm and had two 6 mm diameter holes in the corners of one of the long edges for attachment to *in-situ* support racks.

Exposure of pPVC *In-situ*

Three replicate support racks were constructed on-site at Avecia Biocides, Blackley, Manchester, United Kingdom (see Appendix 1, p146). Each rack consisted of three

lines of 8 mm polypropylene wound rope (Stevecraft, Manchester, United Kingdom), positioned 1.0, 1.5, and 2.0 m above ground level and held between two steel poles set into concrete and distanced 7 m apart. Nylon cable-ties (4 mm width; RS Components, Corby, United Kingdom) were inserted through the rope windings to support pPVC pieces at numbered locations on the rack. Each rack supports up to 300 pPVC pieces which are held free-hanging in order to eliminate fixed-orientation effects. The three racks were positioned in parallel, 1 m apart and were orientated at 60° to the horizontal to limit cross contamination of specimens during rainfall. Ten pieces of pPVC were positioned on each of the three racks at locations chosen using a random number table. At each sampling time, three replicate pPVC pieces were selected at random, one from each rack. Each piece was cut into three 4.2 × 2.5 cm sections and a separate analysis was carried out on each section.

Viable counts of fungi, bacteria and DOA-degrading microorganisms

Plasticized PVC sections (4.2 ×2.5 cm) were placed into 25 ml universal tubes (32 mm diameter, 80 mm length) containing 10 ml sterile distilled H_2O. The tubes were shaken vigorously in an automatic side-arm shaker (Gallenkamp, Leicester, United Kingdom) for one minute. Plasticized PVC samples were transferred to a Petri-dish containing 5 ml H_2O and scraped heavily 3 times on both sides using the flat edge of a sterile scalpel blade. The pPVC, H_2O, and scalpel blade were then returned to the universal tubes and shaken for a further minute using the side-arm shaker. A dilution series to 10^{-3} was prepared from each universal tube. Aliquots of 0.2 ml from each dilution were spread onto three replicate plates of each of malt extract, R2A, and DOA agar. Malt extract agar plates were counted after 5 days of incubation at 25°C; R2A after 7 days and plasticizer agar plates after 14 days. To investigate whether statistically significant changes in CFU counts occurred between sample times, overall mean viable counts for 3 replicate pPVC pieces at each time point were compared using analysis of variance.

Viable counts of fungi within the atmosphere

As a control, to determine whether DOA-degrading organisms could be isolated from the atmosphere, plates of both MEA and DOA agar media were exposed to the atmosphere at a height of 20 m on the roof of the Stopford Building, Oxford Road, Manchester, UK, in March 1997 (United Kingdom winter). Three replicate plates of each medium were retrieved after 20 h of exposure to the atmosphere. The number of

fungal colony forming units (CFUs) on each medium was counted after 3, 5 and 7 days incubation at 25°C.

Scanning electron microscopy (SEM)

Plasticized PVC pieces exposed *in-situ* for 95 weeks were rapidly frozen in liquid nitrogen. Specimens were freeze-dried overnight (BOC Edwards model B5A, Crawley, United Kingdom). Specimens were attached to stubs using Electrodag 915 (Acheson Industries, Reading, United Kingdom) and sputter coated with gold (BOC Edwards model S150) before being examined using a Stereoscan 360 scanning electron microscope (Cambridge Instruments, Cambridge, United Kingdom).

Identification of fungi isolated from pPVC

Fungal isolates were identified by PCR amplification and partial sequencing of the internally transcribed spacer (ITS) regions and the 5.8S rDNA, or of the V3 domain of large subunit (28S) rDNA. Initially only the ITS region was sequenced. However, when this sequence was insufficient to establish identity, the V3 region also was sequenced.

For preparation of genomic DNA, fresh mycelia or yeast cells were harvested in deionized water from overnight plate cultures grown on MEA. The biomass was pelleted by centrifugation at 8000 x g for 10 min and the supernatant discarded. The pellet was frozen by placing the centrifuge tube into liquid nitrogen, and tubes were then stored at -80°C until the pellet was ground in a mortar under liquid N_2. DNA was extracted according to the method of Anderson *et al.* (1996). DNA was observed following electrophoresis in 1% agarose in TPE buffer (90 mM Tris-phosphate, 2 mM EDTA) and staining with ethidium bromide (1 μg ml^{-1} in TPE buffer).

The V3 variable region at the 5' end of the 28S rRNA gene was amplified with the fungal universal primers V3-1 (5'GCATATCAATAAGCGGAGGAAAAG), and V3-2 (5' GGTCCGTGTTTCAAGACGG) (Fell, 1993). PCR reagent concentrations were 0.2 μM for primers V3-1 and V3-2, 2.5 mM $MgCl_2$, 200 μM of each of the 4 dNTPs, and 1.25 units of *Taq* DNA polymerase (Roche Diagnostics Ltd., Lewes, United Kingdom) per 50 μl reaction. Amplification was performed for 30 cycles with denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. ITS regions were amplified using fungal universal primers ITS-1 (5' TCCGTAGGTGAACCTGCGG) and ITS-4 (5' TCCTCCGCTTATTGATATGC) (White *et al.*, 1990). PCR reagent concentrations were as for V3-1/V3-2 with the

exception of 0.25 μM of primers ITS-1 and ITS-4, and 1.5 mM $MgCl_2$. Amplification was performed for 35 cycles with denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min. Amplified products were purified using the QIAquick PCR purification kit (Qiagen Ltd., Crawley, United Kingdom).

Both strands of the amplified products were sequenced using the ABI BigDye Dideoxy Terminator Cycle Sequencing kit (Applied Biosystems Inc., Warrington, United Kingdom). Cycle-sequencing conditions were denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min for 25 cycles, with a final extension at 60°C for 4 min. The annealing temperature was increased to 55°C for sequencing reactions using the V3-1 primer. Forward and reverse sequences were aligned using ABI Autoassembler software (Applied Biosystens Inc.) and overlapping consensus sequence was compared with sequences in the EMBL fungal DNA database (EMFUN) using Fasta3 sequence homology searches. Isolates that were identified using their rDNA sequences were compared with published descriptions of colony and conidial morphology using microscopic examination. A number of isolates, including those for which no rDNA identity was found, were identified using morphological techniques at the International Mycological Institute (IMI), at CABI Bioscience, Egham, United Kingdom.

In-vitro tests for the biodeterioration of pPVC

We characterized the deteriogenic properties of fungi isolated from pPVC and two additional strains of *A. pullulans*. *A. pullulans* IMI70103 was obtained from CABI Bioscience, Egham, United Kingdom, and *A. pullulans* PRAFS8 was provided by Avecia Biocides, Manchester, United Kingdom.

Preparation of inocula. Spores or yeast cells were harvested from MEA plates in deionized water and filtered through 3 layers of lens tissue paper (Whatman, Maidstone, United Kingdom). Suspensions were washed three times in deionized water by centrifugation at 12,000 x g for 8 min, then adjusted to approximately 10^6 spores or yeast cells ml^{-1} in deionized water using a haemocytometer. Suspensions were checked for purity by streaking onto MEA.

Biomass and extracellular esterase production with DOA as the sole carbon source. Isolates were grown in DOA liquid medium for 2 weeks at 25°C and shaken at 250 rpm. Each 100-ml flask, containing 40 ml of medium, was inoculated with 0.2 ml of

spore or yeast suspension. Three replicate flasks were inoculated with each microorganism. For esterase assays, 1.5 ml samples of culture fluid were removed using a syringe, clarified by filtration through a 0.2 μm pore size cellulose-nitrate filter (Sartorius, Epsom, United Kingdom), then either stored at –20°C or used immediately for assays. Cultures of filamentous fungi were then filtered through pre-weighed filter paper (Whatman No. 1) and dried at 70°C to constant weight. Yeast cultures were decanted to pre-weighed 50-ml centrifuge tubes and centrifuged at 3600 x g for 5 min to pellet cells. The supernatant was discarded and tubes were incubated at 70°C to constant weight, usually 24 – 36 h.

Nonspecific esterase activity was determined by a spectrophotometric assay with *p*-nitrophenyl butyrate (PNB) (Sigma) as substrate (Fett *et al.*, 1992). Hydrolysis of PNB yields p-nitrophenol, which absorbs maximally at 400 nm under alkaline conditions. The assay mixture (1 ml volume) contained 2.2 mM PNB in sodium acetate buffer (50 mM, pH 5.5) in cuvettes. Culture fluid (500 μl) was added and cuvettes were incubated at 25°C for 1 hour. The reaction mix was then made alkaline by the addition of 0.75 ml of 0.1 M sodium borate (Sigma) before measurement of absorbance at 400 nm. Esterase activity was determined by reference to a standard curve of p-nitrophenol. A relative extracellular esterase (REE) unit was considered as the enzyme activity that liberates 1 nanomole of *p*-nitrophenol from PNB in 1 hour at 25°C at pH 5.5. Three replicate measurements of the extracellular esterase activity of all fungal isolates were made on separate occasions.

Plasticized PVC weight loss. Pre-weighed pPVC pieces (4.2 x 2.5 cm) were placed into Petri-dishes containing 30 ml MSM liquid supplemented with 0.5 g l^{-1} yeast extract. Three replicate Petri-dishes were inoculated with 0.2 ml spore or yeast suspension from each organism and incubated at 25°C for 6 weeks. Fungal biomass was removed from pPVC pieces by washing in non-ionic detergent (LIP Lipsol, Shipley, United Kingdom) and samples were air-dried at room temperature (21 - 24°C) to constant weight. Statistically significant differences in percentage weight loss relative to control pPVC pieces incubated in sterile medium were determined using analysis of variance.

Clear zone production and colony growth on DOA agar. Clear zone production on agar plates containing emulsified DOA as the sole carbon source was used to test the ability of fungi to degrade DOA plasticizer. Plates containing 20 ml DOA agar were inoculated with 50 μl of spore or yeast suspension placed into 5 mm diameter wells cut at the

center of each plate. Three replicate plates were inoculated for each organism and incubated at 25°C for 14 days. Clear zone production on DOA-agar was scored according to the following criteria: 0, no clearing; 1, faint clearing below colony; 2, clearing extending beyond colony boundary; 3, intense clearing (agar completely transparent) extending beyond colony boundary. Colony growth was reported as follows: 0, no visible growth; 1, slight growth within inoculation well; 2, colony diameter < 2 cm; 3, colony diameter ≥ 2 cm. Tests for clear zone production and growth on DOA agar were replicated on three separate occasions.

Growth using intact pPVC as the sole source of carbon. Plasticized PVC pieces (4.2 x 2.5 cm) were placed on 15 ml of solidified MSM agar in a Petri-dish. A further 10 ml of molten MSM agar, cooled to 45°C, was inoculated with 0.1 ml spore or yeast suspension and poured over the pPVC. Plates were incubated at 25°C for 4 weeks. The following criteria were used to score growth on the pPVC: 0, no visible growth; 1, slight growth, barely visible; 2, growth clearly visible around the edges of the PVC; 3, strong growth visible around edges and in the agar above the pPVC.

rDNA sequences

EMBL accession numbers for rDNA sequences from the 12 fungi identified in this study are as follows (sequences with which matches were made are shown in parentheses): Isolate MZ7, AJ276055 (UO5195); MZ8, AJ276054 (AJ000198); MZ10, AJ276058 (AIY17066); MZ14, AJ276057 (AJ005674); MZ20, AJ276059 (UO5915); MZ58, AJ276062 (AJ244236); MZ65, AJ276061 (AJ244236); MZ95, AJ276060 (AF138904); MZ103, AJ276063 (AF138289); MZ104, AJ276056 (AF033407); MZ107, AJ276065 (AF050278); MZ109, AJ276064 (U94948).

RESULTS

Viable counts of microorganisms colonizing pPVC *in-situ*

Numbers of viable fungi, bacteria and DOA-degrading organisms occurring on pPVC were monitored throughout the *in-situ* trial (Table 1). Fungi were established on the pPVC surface after 40 weeks of exposure (Feb. 1998; United Kingdom winter). No significant increase (P = 0.82) in fungal viable counts occurred during the next 15 weeks, but by week 80 (Jun. 1998) the population had more than doubled. A rapid increase to 1500 ± 220 CFU cm^{-2} occurred in the last 15 weeks of exposure (Dec. – Mar.1988/9; UK winter). CFU counts of fungi able to produce clear zones on DOA agar varied between 65% and 84% of the MEA fungal count.

TABLE 1. Viable counts of all fungi and DOA-degrading fungi isolated from pPVC exposed *in situ* over 95 weeks. The mean fungal CFU count and standard error from three replicate pPVC pieces, one taken from each *in-situ* rack, are shown.

Sample (wks/date)	All fungi on MEA (CFU cm^{-2})	% DOA degrading fungi[a]
10 (July '97)	5 ± 1	74
25 (Oct. '97)	2 ± 0	65
40 (Feb. '98)	110 ± 27	84
55 (Jun. '98)	120 ± 15	76
80 (Dec. '98)	260 ± 27	71
95 (Mar. '99)	1500 ± 220	80

[a]Numbers of fungi recovered on dioctyl adipate (DOA) agar expressed as a percentage of all fungi recovered on malt extract agar (MEA).

Viable counts of fungi within the atmosphere

CFU counts were made on MEA and DOA agar plates exposed to the atmosphere for 20 h. The mean and standard error of fungal counts on plates of MEA and DOA agar were 40 ± 14 and 2 ± 1 CFU respectively. Therefore the proportion of DOA-degrading fungi deposited onto agar plates from the atmosphere was 5%.

Identification of *in-situ* isolates

Fungi with 17 morphologically distinct colony types were identified. rDNA sequence lengths ranged from 496 – 576 bp for the 5.8S rDNA and ITS regions, and from 518 – 562 bp for 28S rDNA. Representative colonies of 10 fungal morphotypes were identified using partial rDNA sequences, and these identifications were confirmed by microscopic examination and comparison with published descriptions of colony and conidial morphology. Unambiguous matches (98 – 100% sequence identity) were made using ITS sequences from isolates MZ8 (*Thanatephorous cucumeris*) (Domsch *et al.*, 1980), MZ10 (*Alternaria infectoria*) (Domsch *et al.*, 1980), MZ58 (*Aureobasidium pullulans*) (Domsch *et al.*, 1980), MZ65 (*Aureobasidium pullulans*) and MZ103 (*Emericella nidulans*; anamorph = *Aspergillus nidulans*) (Domsch *et al.*, 1980), and using the 28S V3 region from isolate MZ109 (*Taphrina deformans*) (Commonwealth Mycological Institute (CMI), 1981). ITS sequence from isolate MZ104 showed 100% identity with *Penicillium glabrum,* and 99.8% identity with *P. lapidosum, P. thomii* and *P. purpurescens,* differing from these species by only a single base pair. However, it was possible to distinguish MZ104 as *P. glabrum* on the basis of published colony morphology descriptions (Pitt, 1979). ITS sequences from isolates MZ7 and MZ20 (both *Alternaria alternata*) showed the same level of identity (99.63%) with *Alternaria lini* as well as with *Alternaria alternata* and we were unable to differentiate these species following morphological examination. As *A. alternata* is an extremely common species found in abundance in soil and on other substrates (Ellis, 1971), while *A. lini* is relatively rare (Rotem, 1994) and may be contained within *A. alternata* (McKay *et al.*, 1999), isolates MZ7 and MZ20 were named as *A. alternata*. The ITS sequence of MZ14 had a relatively low (95%) identity with *Petromyces muricatus*. However, *P. muricatus* is a teleomorphic species of the *Aspergillus ochraceus* group (Domsch *et al.*, 1980; Varga *et al.*, 2000), and the colony and conidial morphology of MZ14 were identical to published descriptions of *A. ochraceus* (Raper & Fennel, 1965). Thus MZ14 was named as *Aspergillus ochraceus*. The 28S rDNA sequence from isolate MZ107 showed 95% identity with *Phaeococcomyces nigricans*. As species level identification requires further biochemical characterization, MZ107 was named only to genus level.

Seven fungal morphotypes, including isolates MZ108 and MZ110 for which no ITS or 28S rDNA match was available, were identified at the International Mycological Institute, Egham, United Kingdom using morphological techniques. Identification to species level was possible for the yeasts MZ108 (*Kluyveromyces marxianus*) and

MZ110 (*Rhodotorula aurantica*), and the filamentous fungi MZ111 (*Epicoccum nigrum*), and MZ112 (*Paecilomyces lilacinus*). MZ113 was identified only to genus level (*Kluyveromyces sp.*), and MZ114, an intensely red yeast, could not be identified.

In-situ colonization sequence

We made viable counts of the 17 fungal morphotypes throughout the colonization period (Table 2). *Aureobasidium pullulans* was the primary colonizing isolate and was dominant between 25 and 80 weeks of exposure. After 25 weeks, *A. pullulans* was isolated from only two of the three *in-situ* racks, but by 40 weeks this fungus was established on all three racks and its frequency had increased significantly (Table 2) ($P = 0.02$). The *A. pullulans* mean viable count was stable from 40 to 55 weeks ($P = 0.46$), but increased significantly from 55 to 80 weeks and again from weeks 80 – 95 (Table 2). After 95 weeks, larger colonies of *A. pullulans* were visible to the eye as black specs ($\leq$ 2mm in diameter) on the pPVC substratum.

A group of yeasts and yeast-like fungi established after 80 weeks of exposure. These microorganisms had colony morphologies identical to isolates MZ107 (*Phaeococcomyces* sp.), MZ108 (*Kluyveromyces marxianus*), MZ109 (*Taphrina deformans*), MZ110 (*Rhodotorula aurantica*), and MZ114 (unidentified red yeast). A significant increase in the numbers of each of these organisms occurred on all three racks between 80 and 95 weeks of exposure (P values ≤ 0.005) (Table 2). The most abundant of these isolates had the same colony morphology as MZ109. *Epicoccum nigrum*, a filamentous fungus also appeared to be a secondary colonizer. It was initially isolated after 80 weeks from one of the *in-situ* racks, but by 95 weeks this organism was recovered from all three racks (Table 2).

Throughout the *in-situ* trial several filamentous fungi, and the yeast *Kluyveromyces marxianus,* occurred sporadically (0 – 15 CFU cm^{-2}). The filamentous fungi included representatives of the genera *Alternaria, Aspergillus, Cladosporium, Emericella, Paecilomyces, Penicillium*, and *Thanatephorous*. Usually these organisms were isolated from only one or two of the *in-situ* racks during sampling, although *Alternaria alternata* was recovered in low numbers from all three racks after 10 and 40 weeks of exposure (Table 2).

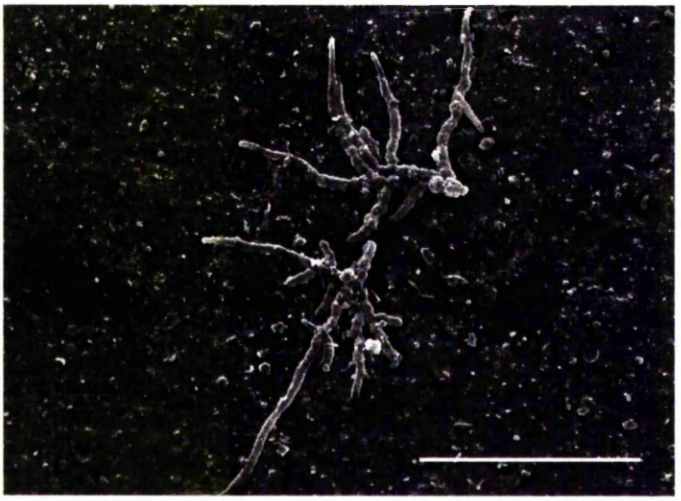
TABLE 2. Frequency of occurrence of fungi with different colony morphologies recovered from pPVC throughout the in-situ trial. The mean viable count and standard error from three replicate pPVC pieces, one taken from each *in-situ* rack, is shown. The number of racks on which fungi occurred at each sample time is indicated in parentheses.

Colony Morphotype	Time (wks)	(Mean CFU cm^{-2})				
	10	25	40	55	80	95
Aureobasidium pullulans	0	1 ± 1(2)	93 ± 50(3)	120 ± 35(3)*	150 ± 20(3)	390 ± 53(3)
Epicoccum nigrum	0	0	0	0	0.3 ± 0.7(1)	32 ± 13(3)
Phaeococcomyces sp.	0	0	0	0	10 ± 9(2)	64 ± 49(3)
Pink-red yeast. Unidentified.	0	0	0	0	17 ± 11(3)	170 ± 110(3)
Rhodotorula aurantica	0	0	0	0	0.7 ± 0.7(3)	73 ± 69(3)
Taphrina deformans	0	0	0	0	86 ± 55(3)	490 ± 240(3)
Kluyveromyces sp.	0	0	0	0	0	220 ± 153(3)
Alternaria alternata	3 ± 1(3)	0.3 ± 0.6(1)	4 ± 3(3)	0	0	4 ± 7(1)
Alternaria infectoria	0	0	0.2 ± 0.4(1)	0	0.1 ± 0.3(1)	0
Aspergillus niger	0.3 ± 0.5(1)	0	0	0	0	7 ± 11(2)
Aspergillus ochraceus	0	0	3 ± 5(1)	0	0	0
Cladosporium herbarum	0	0	0	0	0	0.6 ± 0.7(2)
Emericella nidulans	2 ± 2(2)	0.3 ± 1.2(1)*	0	0	0.1 ± 0.3(1)	0
Kluyveromyces marxianus	0	0	0	0	0.6 ± 1.1(1)	0
Paecilomyces lilacinus	0	0	0	0	0	6 ± 7(2)
Peniciilium glabrum	0.5 ± 1.2(1)	0	12 ± 22(1)	0	0	0
Thanatephorous cucumeris	0	0	0	0	0.3 ± 0.5(2)	3 ± 5(1)

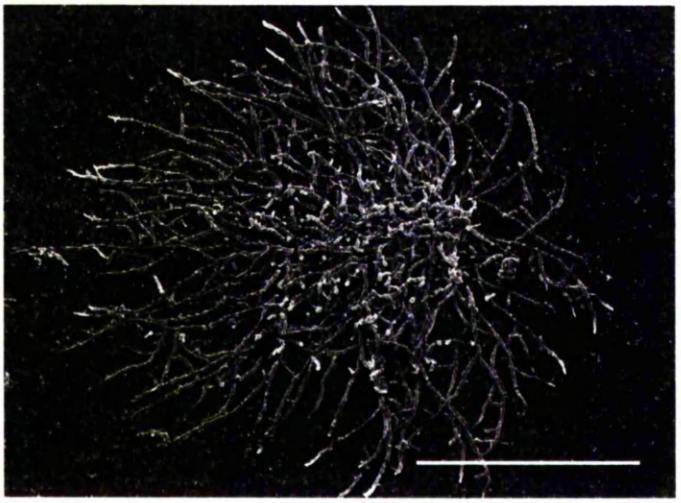
*No statistically significant change in mean CFU count in comparison with previous sample time ($P > 0.05$).

SEM of pPVC samples exposed *in-situ*

pPVC samples exposed to the atmosphere for 95 weeks were examined under SEM (Fig. 1). Colonies of *A. pullulans*, 50 - 2000 μm in diameter, were randomly dispersed across the surface of the pPVC. *A. pullulans* appeared both as young colonies in the early stages of development (Fig. 1a) and as well established, circular or oval shaped colonies with extensive hyphal growth (Fig. 1b). Yeast-phase growth of *A. pullulans* was not observed and mycelia appeared as chains of branching, septate hyphae. There was no evidence of penetration of the pPVC substratum by hyphae of *A. pullulans.* Very few other microorganisms were observed on the pPVC under SEM. Ovoid, yeast-like



a)



b)

Fig. 1. SEM of the surface of pPVC exposed *in*-situ for 95 weeks. a) *A. pullulans* colony in early stages of development; Bar = 100 µm, b) Established *A. pullulans* colony; Bar = 200 µm.

cells occurred occasionally either singly or in clumps of 2-3 cells, both associated with *A. pullulans* colonies and on uncolonized areas of the plastic. No bacteria were observed.

In-vitro tests for the biodeterioration of pPVC

Organisms with the highest level of extracellular esterase activity (100 - 250 REE ml^{-1}) included all three strains of *A. pullulans*, MZ10 (*Alternaria infectoria*), MZ95 (*Aspergillus niger*), MZ107 (*Phaeococcomyces sp.*) and MZ111 (*Epicoccum nigrum*). Isolates with little or no extracellular esterase activity ($\leq$ 2.0 REE ml^{-1}) included MZ110 (*Rhodotorula aurantica*), MZ114 (unidentified yeast), MZ103 (*Emericella nidulans*) and MZ8 (*Thanatephorous cucumeris*) (Table 3).

Extracellular esterase activity was not correlated with activity in other tests. No significant difference (P = 0.15) occurred between the mean esterase activity of isolates that produced strong DOA clear zones (Score 3; MZ7, MZ10, MZ58 and MZ20) and those that demonstrated no clearing (Score 0; MZ95, MZ104, MZ107, MZ108, MZ110, MZ114 and MZ115). Similarly, there was no significant difference (P = 0.28) in mean esterase activity between isolates showing strong growth on DOA agar (Score 3; MZ7, MZ10, MZ14, MZ20, MZ95, MZ111, MZ112 and MZ115) and those showing no growth (Score 0; MZ107, MZ108, MZ110 and MZ114). We also observed differences between strains of the same fungal species. For example, *A. alternata* strain MZ20 showed poor esterase activity in comparison with *A. alternata* strain MZ7. Measurements of extracellular esterase activity, clear zone production and growth on DOA agar were carried out on three separate occasions and the same trends among all of the fungal isolates were observed.

The ability of *in-situ* isolates to cause weight loss from pPVC was determined after incubation with pPVC for 6 weeks under MSM supplemented with yeast extract The net weight loss in all cases was very low (7 – 50 mg), and therefore direct comparisons of the weight loss caused by individual species were not possible. However, all 20 isolates tested caused significant ($P \leq$ 0.01) weight losses of greater than 1% in comparison to control pPVC pieces incubated in sterile medium (Table 3). The greatest weight reduction of 6.8% was caused by MZ103 (*E. nidulans*).

TABLE 3. Deteriogenic properties of fungi isolated from pPVC during the *in-situ* trial. Test methods used were measurement of extracellular esterase activity and biomass production during growth using DOA as the sole carbon source, clear zone production and growth on DOA-agar, growth on pPVC and pPVC weight loss. Isolates are ranked in order of decreasing extracellular esterase activity.

Isolate	Identification	Esterase activity (REE[a] ml^{-1})	Biomass in DOA liquid medium (mg/40 ml)	Specific esterase activity (REE[a] mg^{-1})	Growth on DOA agar[b]	Clearing on DOA agar[c]	Growth on pPVC[d]	pPVC weight loss (%)
MZ58	*Aureobasidium pullulans*	250 ± 40	10.7 ± 4.1	1500 ± 1267	1	3	1	3.7 ± 0.7
MZ10	*Alternaria infectoria*	240 ± 17	11.9 ± 0.3	800 ± 75	3	3	2	3.9 ± 0.5
MZ95	*Aspergillus niger*	190 ± 7	13.0 ± 2.8	630 ± 210	3	-	1	1.8 ± 0.2
PRA FS8	*Aureobasidium pullulans*	160 ± 6	8.8 ± 1.5	760 ± 240	1	2	3	3.6 ± 0.7
IMI70103	*Aureobasidium pullulans*	150 ± 16	5.2 ± 1.5	1300 ± 700	2	2	2	3.4 ± 0.7
MZ107	*Phaeococcomyces sp.*	120 ± 4	6.9 ± 2.4	840 ± 370	-	-	3	4.0 ± 0.2
MZ111	*Epicoccum nigrum*	100 ± 19	23 ± 4.2	180 ± 44	3	2	2	1.4 ± 0.3
MZ7	*Alternaria alternata*	81 ± 10	17 ± 0.4	300 ± 120	3	3	2	4.1 ± 0.3
MZ109	*Taphrina deformans*	68 ± 10	27 ± 9.6	160 ± 130	1	1	1	1.4 ± 0.4
MZ 113	*Kluyveromyces sp.*	51 ± 19	18 ± 11.7	260 ± 260	1	1	1	1.9 ± 0.7
MZ115	*Cladosporium herbarum.*	50 ± 21	14 ± 4.2	150 ± 78	3	-	3	1.7 ± 0.4
MZ112	*Paecilomyces lilacinus*	27 ± 9	44 ± 7.0	26 ± 11	3	2	3	4.0 ± 1.5
MZ108	*Kluyveromyces marxianus*	18 ± 6	25 ± 8.7	49 ± 51	-	-	-	1.8 ± 0.2
MZ14	*Aspergillus ochraceus*	12 ± 5	17 ± 1.3	29 ± 8.4	3	2	1	3.9 ± 0.3
MZ20	*Alternaria alternata*	11 ± 6	12 ± 2.3	27 ± 14	3	3	2	4.2 ± 1.2
MZ104	*Peniciilium glabrum*	5 ± 3	31 ± 5.3	8.1 ± 6.4	1	-	1	2.0 ± 0.4
MZ8	*Thanatephorous cucumeris*	2 ± 1	11 ± 0.6	6.1 ± 2.3	2	2	-	1.7 ± 0.1
MZ 114	Pink-red yeast. Unidentified.	0.4 ± 0.3	17 ± 3.6	1.3 ± 1.4	-	-	-	1.8 ± 0.4
MZ103	*Emericella quadrilineata*	0.4 ± 0.3	7.3 ± 3.2	6.5 ± 8.3	1	1	1	6.8 ± 0.6
MZ110	*Rhodotorula aurantica*	0.3 ± 0.4	16 ± 4.5	1.0 ± 1.5	-	-	-	1.9 ± 0.2

Mean values ± 1 SE of mean are shown. [a]A relative extracellular esterase (REE) unit is the enzyme activity that liberates 1 nanomole of *p*-nitrophenol from *p*-nitrophenyl butyrate in 1 hour at 25°C and at pH 5.5. [b]Growth on DOA agar: 0, no visible growth; 1, slight growth within inoculation well; 2, colony diameter < 2 cm; 3, colony diameter [c]Clear zone production on DOA agar: 0, no clearing; 1, faint clearing below colony; 2, clearing extending beyond colony boundary; 3, intense clearing (agar completely transparent) extending beyond colony boundary. [d]Growth on pPVC: 0, no visible growth; 1, slight growth, barely visible; 2, growth clearly visible around the edges of the PVC; 3, strong growth visible around the edges and in the agar above the pPVC. [e]The initial weight of individual pPVC pieces (100% weight loss value) ranged from 500 to 730 mg. The mean weight loss from sterile pPVC controls was 0.1 ± 0.1%.

DISCUSSION

This study is the first detailed, quantitative investigation of the microbial colonization of pPVC. *Aureobasidium pullulans* was the principal colonizing fungus. This organism initially colonized the pPVC after 25 weeks of exposure to the atmosphere and was isolated throughout the remainder of the *in-situ* trial. SEM studies demonstrated that *A. pullulans* colonized pPVC in the absence of other microorganisms and therefore acts as a primary colonizer of pPVC. *A. pullulans* is increasingly recognized as the major causative agent of defacement of various diverse materials, such as painted surfaces (Goll & Coffery, 1948; Cooke, 1959; Winters *et al.*, 1976) and wood (Kaarik, 1974), in addition to pPVC exposed to tropical conditions (Hamilton, 1983; Upsher & Roseblade, 1974). The present study demonstrates that *A. pullulans* is also an important colonizer of pPVC in temperate climates.

The success of *A. pullulans* in colonizing pPVC *in-situ* is probably due to a combination of several factors. *A. pullulans,* which usually colonizes the phylloplane (Domsch *et al.*, 1980), can withstand periods of desiccation and high temperatures, and produces highly melanized hyphae that protect against UV exposure (Crang & Pechak, 1978). *A. pullulans* also produces extracellular polysaccharides which may facilitate permanent adhesion to surfaces (Andrews *et al.*, 1994), and factors controlling adhesion of *A. pullulans* to pPVC have recently been characterized (Webb *et al.*, 1999). Therefore adaptations for survival within the phylloplane probably confer advantages on *A. pullulans* for the colonization of painted and plastic surfaces within the environment (Zabel & Terracina, 1979).

A. pullulans also has considerable enzymatic capabilities. All three strains of *A. pullulans* produced high levels of extracellular esterase and could degrade DOA *in-vitro*. In addition to extracellular esterase, *A. pullulans* also produces significant amounts of cellulase, proteinase, phosphatase, invertase and maltase (Winters & Guidetti, 1976). The ability of *A. pullulans* to secrete such a variety of hydrolytic enzymes might enable it to utilize exogenous carbon sources that accumulate on the pPVC during long periods of exposure *in-situ*. Extracellular esterase production is hypothesised to aid in the colonization of pPVC through the hydrolysis of organic-ester plasticizers (Berk *et al.*, 1957; Klausmeier & Jones, 1961; Mills & Eggins, 1974). However, in this study extracellular esterase production did not correlate with DOA clearing or growth using pPVC as the sole source of carbon. These results may be due

to differences in the specificity of esterase enzymes towards DOA plasticizers and the p-nitrophenyl butyrate synthetic substrate used in esterase assays. Thus measurement of esterase activity alone is not a reliable indicator of the ability of an organism to degrade plasticizers or colonize pPVC.

A group of yeasts and yeast-like fungi established in on the pPVC much later that *A. pullulans*, towards the end of the *in-situ* trial. Therefore these yeasts probably play a secondary role in the colonization of pPVC in the sense that they require additional nutrients, e.g. the metabolites of other fungi, or the accumulation of exogenous nutrients, before they can grow on the pPVC. These hypotheses are consistent with the observation that none of the yeasts, except *Kluyveromyces sp.*, can degrade DOA or grow on pPVC as the sole source of carbon *in-vitro*. Yeasts are not generally considered as important deteriogenic organisms on artificial surfaces, even though they have been recovered from deteriorated rubber and building materials (Lodder & Krejer-van Rij, 1967), and from deteriorated pPVC during tropical exposure trials in Puerto Rico (Hamilton, 1983). Neither study provided information on the abundance of the yeasts or their role in the biodeterioration of these materials. Thus, the present study is the first to attribute a significant role to yeasts in the colonization of pPVC.

Interestingly, only a few yeast cells were observed during SEM studies of the pPVC substratum. These results suggest that high yeast CFU counts resulted from a small number of rapidly multiplying yeast colonies, and highlight the general problem of using CFU counts to quantify fungi. CFU counts depend on how readily the fungal material on the pPVC breaks into individual propagules during the isolation procedure. For example, for the same amount of biomass, a colony of a budding yeast or a sporulating filamentous fungus may yield many more CFUs than a spreading hyphal mycelium. Thus, while CFU counts are useful in determining which organisms are colonizing and multiplying on the pPVC, they are not usually a reliable indicator of the fungal biomass present on the substratum.

Bacteria were not isolated from the pPVC during the trial and none were detected during SEM studies or following DAPI-fluorescence staining of organisms removed from the pPVC substratum (data not shown). Bacterial growth might be inhibited by desiccation, solar irradiation, or *in-situ* acidification of the pPVC following photochemical or thermal degradation (Hollande & Laurent, 1997; Martinez *et al.*,

1996). Acidification of the pPVC could inhibit bacterial growth as fungi tolerate lower pHs than do bacteria.

Fungal colonization of pPVC appeared to be influenced by seasonal climatic changes. Major increases in fungal CFU counts occurred only during the British winter. For example, fungal CFU counts increased by 470% during the winter period between Nov. 1998 (80 weeks) and Mar. 1999 (95 weeks), largely due to the establishment of yeasts and yeast-like fungi on the pPVC. In contrast, no increases in fungal CFU counts occurred when pPVC pieces were sampled during the British summer months. Fungal growth or sporulation during the summer period might be inhibited by desiccation and high temperatures caused by long periods of direct solar irradiation. Indeed, Upsher & Roseblade (1984) reported that a marked reduction in the amount of fungal growth on pPVC can occur during dry periods. Extended studies of the colonization process are needed to determine whether yeasts that establish on the pPVC during the winter months can survive on the pPVC through the succeeding summer period.

Whether an organism can colonize pPVC probably also depends on its ability to obtain carbon from the pPVC. Evidence supporting this hypothesis comes from the observation that between 64% and 84% of fungi colonizing pPVC could degrade DOA. In contrast, among fungi deposited onto agar plates from the atmosphere, only 5% could degrade DOA at the time of sampling. Although the proportion of DOA-degrading fungi within the atmosphere is likely to be influenced by environmental parameters and probably varies seasonally, these results support the hypothesis that enrichment of fungi able to degrade DOA occurs on the pPVC substratum and that organisms that can degrade DOA may have a competitive advantage during the colonization of pPVC.

In addition to *A. pullulans*, many of the recovered fungi, e.g. *Alternaria spp.*, *Aspergillus spp.*, *Paecilomyces sp.* and *Cladosporium sp.* have previously been isolated from deteriorated pPVC in tropical exposure trials (Hamilton, 1983; Roberts & Davidson, 1986), and are common colonizers of painted surfaces and building materials (for a review, see Gaylarde & Morton, 1999). In particular, *A. alternata*, *A. infectoria* and *Paecilomyces lilacinus* had high activity in all of the test methods, demonstrating that they are potentially important degraders of pPVC within the environment. Under ideal growth conditions within warm and humid tropical exposure trials, these organisms would probably grow readily on the pPVC substratum. However, these organisms were isolated infrequently and in low numbers in this study, suggesting that

environmental factors limited the establishment of these organisms on the pPVC. Thus, while *in-vitro* methods identified fungi potentially capable of causing biodeterioration of pPVC, they were not predictive of the organisms that colonized pPVC in the environment.

We observed discrepancies between the ability of fungi to grow on the intact pPVC formulation as the sole carbon source and their ability to degrade DOA *in-vitro.* For example, *Phaeococcomyces sp.* grew well on pPVC but could not produce clear zones in DOA-agar. It is possible that components of the pPVC formulation other than the plasticizers can support the growth of fungi on pPVC. We used DOA in the present study because it is known to be more susceptible than the other plasticizer, DOP, to microbial attack (Berk *et al.*, 1957). However, in addition to DOA and DOP the pPVC formulation also contains small quantities of a calcium-zinc stearate stabilizer and an epoxidized oleate ester stabilizer that can be utilized by various fungi as a sole carbon source (Roberts & Davidson, 1986). Thus, while the homogenized plasticizer agar technique is useful to determine if an organism can degrade a plasticizer, it is not necessarily predictive of the organism's ability to grow on a complex pPVC formulation.

We found no evidence that the mechanical properties of the pPVC were altered due to microbial degradation of plasticizers in the environment. Although fungi can increase the tensile strength of pPVC *in-vitro* due to the degradation of plasticizers (Seal & Pantke, 1988; Whitney, 1996), tensile testing of exposed pPVC pieces showed no significant change in either the tensile strength or the % elongation at breaking (see Appendix 2, p147). We think that the fungal biomass that accumulated on pPVC pieces during the *in-situ* trial was insufficient to cause a measurable change in the mechanical properties of the pPVC.

In summary, our results suggest that a colonization sequence may occur during colonization of pPVC *in-situ. A. pullulans* is the principal colonizing fungus and secondary colonizing yeasts establish much later. *In-vitro* biodeterioration tests were not predictive of the ability of fungi to colonize pPVC in the environment, emphasizing the importance of field trials in investigations of the microbial susceptibility of pPVC formulations. Knowledge of the organisms that colonize pPVC and their ecology is essential for the design of novel pPVC formulations and biocides that provide long-term protection against biodeterioration of pPVC *in-situ.*

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Chapter 3.

Adhesion of the deteriogenic fungus Aureobasidium pullulans to PVC

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ABSTRACT

Initial adhesion of fungi to plasticized polyvinyl chloride (pPVC) may determine subsequent colonization and biodeterioration processes. The deteriogenic fungus *Aureobasidium pullulans* was used to investigate the physicochemical nature of adhesion to both unplasticized PVC (uPVC) and pPVC containing the plasticizers dioctyl phthalate (DOP) and dioctyl adipate (DOA). A quantitative adhesion assay using image analysis identified fundamental differences in the mechanism of adhesion of *A. pullulans* blastospores to these substrata. Adhesion to pPVC was greater than that to uPVC by a maximum of 280% after a 4 h incubation with 10^8 blastospores ml^{-1}. That plasticizers enhance adhesion to PVC was confirmed by incorporating a dispersion of both DOA and DOP into the blastospore suspension. Adhesion to uPVC was increased by up to 308% in the presence of the dispersed plasticizers. Hydrophobic interactions were found to dominate adhesion to uPVC because *i*) A strong positive correlation was observed between substratum hydrophobicity (measured by using a dynamic contact angle analyzer) and adhesion to a range of unplasticized polymers including uPVC, and *ii*) neither the pH nor the electrolyte concentration of the suspension buffer, both of which influence electrostatic interactions, affected adhesion to uPVC. In contrast, adhesion to pPVC is principally controlled by electrostatic interactions. Enhanced adhesion to pPVC occurred despite a relative reduction of 13° in the water contact angle of pPVC when compared to uPVC. Furthermore, adhesion to pPVC was strongly dependent on both the pH and electrolyte concentration of the suspension medium, reaching maximum levels at pH 8 and with an electrolyte concentration of 10 mM NaCl. Plasticisation with DOP and DOA therefore increases adhesion of *A. pullulans* blastospores to pPVC through an interaction mediated by electrostatic forces.

INTRODUCTION

Major problems of substratum damage occur when plasticized polyvinylchloride (pPVC) is colonized by microorganisms in many different environmental situations. It has long been established that this susceptibility results from the presence of plasticizers, commonly organic acid esters such as dioctyl phthalate (DOP) and dioctyl adipate (DOA), added to modify physical or mechanical properties of the polymer (Brown, 1945). Since these early studies, degradation of ester-based plasticizers has been demonstrated among both bacteria (Booth *et al.*, 1968; Booth & Robb, 1968; Eaton & Ribbons, 1982) and fungi (Berk *et al.*, 1957; Whitney, 1996). Loss of plasticizers from pPVC due to microbial degradation results in brittleness, shrinkage, and ultimately failure of the PVC in its intended application.

Although no detailed quantitative studies have been published, fungi are reported to be the principal deteriogenic organisms in structural and outdoor applications of pPVC (Bessems, 1988; Hamilton, 1983). However, despite widespread commercial use of pPVC and considerable economic losses due to its biodeterioration, mechanisms of fungal attachment to pPVC have not previously been examined.

Microbial adhesion is the first in a series of events that occur during the colonization of a solid substratum. Adhesion to inert materials such as plastics or glass is known to be controlled by nonspecific interactions between the cell surface and the substratum. Research has focused on bacterial adhesion, where hydrophobicity both of the substratum (Wiencek & Fletcher, 1987; Busscher *et al.*, 1990; Rönner *et al.*, 1990) and of the cell surface (Pascual *et al.*, 1986; van Loosdrecht *et al.*, 1987b; Stenstrom, 1989) and electrostatic charge on the cell surface (van Loosdrecht *et al.*, 1987a; Gilbert *et al.*, 1991; Rijnaarts *et al.*, 1995) are important factors in adhesion to inert substrata.

While bacterial adhesion to surfaces has be studied extensively, the nonspecific adhesion of fungi has received comparatively little attention. Most fungal adhesion studies have focused on adhesion of the opportunistic pathogen *Candida albicans* to synthetic materials used for medical prostheses. Increased adhesion of *C. albicans* to plastics has been associated with increased hydrophobicity both of the fungal surface (Miyake *et al.*, 1986; Panagoda *et al.*, 1998) and of the substratum (Klotz *et al.*, 1985). Electrostatic forces have also been demonstrated to influence adhesion of *C. albicans* to hydrophilic glass (Jones & O'shea, 1994), although are thought to be of minor importance in adhesion to more hydrophobic plastics (Klotz *et al.*, 1985). Therefore

similar physicochemical characteristics appear to control both adhesion of bacteria and *C. albicans*.

Nothing is known about the physicochemical factors controlling adhesion of fungi that colonize and deteriorate plastics within the environment. This study reports on the mechanisms of adhesion of the deuteromycete *A. pullulans* because it was found to be the dominant fungus causing deterioration of pPVC films during outdoor exposure trials in Florida (Hamilton, 1983). *A. pullulans* is ubiquitous within the environment and is known to colonize many habitats (Cooke, 1959). It is one of relatively few fungi that can colonize living leaf surfaces (Andrews *et al.*, 1994) and is also the principal colonizer of painted wood surfaces (Eveleigh, 1961).

As part of a long term study of microbial colonization processes occurring on pPVC, the initial adhesion of the deteriogenic fungus *A. pullulans* has been studied *in vitro*. We have investigated the effect of incorporating the plasticizers DOP and DOA into PVC on the adhesion of *A. pullulans* blastospores. Physicochemical parameters influencing the nonspecific adhesion of blastospores to both uPVC and pPVC have also been investigated.

MATERIALS AND METHODS

Aureobasidium pullulans (de Bary) Arnaud

A. pullulans (IMI70103) was maintained on malt extract agar (Oxoid, Unipath Ltd., Basingstoke), and to produce blastospores, cultures were incubated for 5 days at 25°C in the dark. Under these conditions, only the mycelial and blastospore morphotypes of *A. pullulans* developed. For long term storage, blastospores were frozen at -80°C in 20% (vol/vol) glycerol solution (BDH, Poole).

Growth of *A. pullulans* on pPVC as the sole carbon source was used as a test method to confirm the ability of this isolate to deteriorate pPVC. A piece of pPVC (4 by 4 cm) was sandwiched between two layers of 12 g l^{-1} bacteriological agar (Oxoid) in de-ionized water. The upper layer of agar was seeded with approximately 1×10^5 blastospores ml^{-1} of *A. pullulans* before pouring. A control agar plate was prepared that contained blastospores but without a piece of pPVC incorporated. Both plates were incubated at 25°C for 7 days, after which time growth of *A. pullulans* could clearly be seen in agar immediately above the pPVC. Absence of growth on the control plate confirmed the ability of *A. pullulans* to obtain a source of carbon from the pPVC.

Adhesion assay

pPVC sheets (0.5 mm thick) were formulated that contained the following (parts per hundred resin): EP 6779 (PVC resin) (European Vinyls Corporation (UK) Ltd.), 75; Vinnolit C65V (PVC resin), 25; DOA (plasticizer) (Exxon Chemicals Ltd.), 25; DOP (plasticizer) (Exxon Chemicals Ltd.), 25; Lankromark LN138 (calcium-zinc stabilizer) (AKCROS), 2; and Lankroflex ED63 (epoxidized oleate ester), 3. uPVC sheets (0.2 mm thick) were obtained from Goodfellow Ltd., Cambridge. Discs, 4 mm in diameter, were cut from sheets using a pair of punch-pliers (RS components). Discs were cleaned in 2% Lipsol detergent (LIP, Shipley, England) and rinsed thoroughly in de-ionized water. Handled by the edges only and with forceps, discs were placed in rows of five into the wells of a 96-well assay plate (Costar).

A. pullulans blastospores were harvested in phosphate-buffered saline (PBS) containing 8.0 g l^{-1} NaCl, 0.2 g l^{-1} KCl, 1.15 g l^{-1} Na_2HPO_4, 0.2 g l^{-1} KH_2PO_4, and 0.1 g l^{-1} $MgCl_2$ at pH 7.3 and separated from hyphae by filtration through three layers of lens tissue paper. Blastospores were centrifuged for 8 min at 3600 × g, washed three times and resuspended in PBS to an optical density at 540 nm of 0.59 (10^8 blastospores ml^{-1}).

Aliquots of 200 µl of the blastospore suspension were placed into wells containing pPVC discs by using a multi-channel pipette (Treff Lab). Following incubation at 25°C, the blastospore suspension was removed by pipette and discs were washed three times by sequentially adding and removing 200 µl aliquots of PBS. During the washing procedure, pipette tips were inserted into the base of wells so that liquid crossed the surfaces of discs evenly and with minimal variation in shear forces. Spores were then fixed for 15 min by the addition of 100 µl of 20% (vol/vol) formaldehyde to each well and washed once in PBS. Discs were allowed to air dry for 1 h before being stained with Gram crystal violet (Difco). Stain (100 µl) was applied to each well for 5 s and quickly removed, and discs were washed a further three times in PBS. Stained discs were then transferred from wells to a microscope slide for image analysis.

Blastospores on pPVC discs were visualized by using a Leica Medilux microscope equipped with an automated stage for image analysis. Digital images of the pPVC surface were captured under bright field illumination with a charged-coupled device (CCD) camera (Sony XC-75CE) and the percentage of surface covered with attached blastospores was quantified with image analysis software (Quantimet Qwin 570, Version 01.00, Leica, Cambridge Ltd.).

Reproducibility of adhesion data

To determine disc to disc (intrabatch) variation within the adhesion assay, rows of 5 discs of both uPVC and pPVC were exposed to a suspension of 10^8 blastospores ml^{-1} derived from a single plate. Five rows were sampled for each material and adhesion was quantified after a 4 h incubation period. To determine whether different batches of blastospores gave different levels of adhesion (interbatch variation), adhesion was measured by exposing rows of 5 pPVC and uPVC discs to blastospores harvested from 5 different cultures. Inter- and intrabatch variation in adhesion were statistically assessed by using analysis of variance.

Kinetics of adhesion to uPVC and pPVC

To determine the time period required for maximal adhesion to pPVC, a time course experiment was carried out over a 10 h period using a suspension of 10^8 blastospores ml^{-1}. After each sample time, 5 pPVC discs were removed, washed, and stained, and the mean percentage surface cover with blastospores for the discs was determined. The influence of blastospore concentration on numbers of blastospores attaching was

determined by exposing rows of 5 discs to blastospore concentrations in the range 2×10^7 to 5×10^8 blastospores ml^{-1} for a 4 h adhesion period.

Influence of plasticizers on adhesion to uPVC

A mixed dispersion of both DOP and DOA was created within PBS suspension buffer to examine the influence of plasticizers on adhesion of *A. pullulans* blastospores to uPVC. Five ml of each plasticizer was added to 400 ml of PBS. Both DOP and DOA are immiscible with water but are completely miscible with each other and form an organic phase over the PBS. The entire volume was homogenized (Ystral D-7801, 260 W, 25,000 rpm, Dotttingen) for 1 min and centrifuged for 10 min at 3,600 × g in order to remove large droplets of plasticizer. The resulting dispersion was separated from liquid DOP and DOA remaining on the surface of the PBS by running the volume through a glass separation funnel fitted with a tap. The eluent from the column was considered to be the 100% plasticizer concentration. Dilutions were prepared to contain relative plasticizer concentrations in the range 0 to 100% of this undiluted dispersion. Blastospores were suspended to 1×10^8 blastospores ml^{-1} in each dilution of the dispersion and applied to rows of 5 discs of uPVC for 4 h. Plasticizer concentrations in PBS were kept constant throughout the washing procedure.

Both DOP and DOA exhibit low water solubility of up to 1 mg l^{-1} H_2O (Chemical Abstract Service Nos. 117-84-0 for DOP and 103-23-1 for DOA). To determine the effect of low levels of dissolved plasticizer on adhesion, dispersed plasticizers in PBS were ultracentrifuged at 80,000 × g for 30 min. to remove undissolved plasticizer. The resulting clear solution was separated from remaining liquid DOP and DOA using a separating funnel and the eluent from the column was considered to be the 100% dissolved plasticizer concentration. Dilutions were prepared to contain relative dissolved plasticizer concentrations in the range 0 – 100% of the undiluted solution. Rows of 5 discs of uPVC were exposed for 4 h to blastospores suspended to 10^8 blastospores ml^{-1} in each dilution. Dissolved plasticizer concentrations in PBS were kept constant throughout the washing procedure.

Effect of substratum hydrophobicity on adhesion

Contact angle measurements were made on a range of polymers and glass to examine the influence of substratum hydrophobicity on adhesion. The polymers used were polyethylene tetraphthalate (PET), polypropylene (PP), polytetrafluoroethylene (PTFE) and unplasticized polyvinyl chloride (uPVC) (Goodfellow Ltd., Cambridge),

polyethylene (PE) and fluoroethylenepropylene (FEP) (Fluorplast, Raamsdonkveer, the Netherlands), and tissue culture-treated polyethylene tetraphthalate (TC-PET; Thermanox) (Agar Scientific Ltd., Stansted). Glass microscope slides were obtained from Chance Proper Ltd., Warley, England.

Surface hydrophobicity of materials was determined using a dynamic contact angle analyzer (model DCA-312, Cahn Instruments, Madison, Wis.) (Domingue, 1990). This equipment uses the principle of the Wilhelmy balance and has an advantage over sessile drop methods in that larger surface areas may be sampled (Lander *et al.*, 1993). Samples (2 × 2 cm) were cut from sheets of each polymer and the contact angle measured by immersing to a depth of 1 cm at a stage speed of 19.6 μm s^{-1}. The wetting fluid used was de-ionized water (Elix 3, Millipore Corp., Watford). Advancing contact angles (θ_a) were determined on five replicate samples of each material.

Six replicate discs of each polymer were prepared as described for pPVC and uPVC. Fragments of glass small enough to be inserted into plate-wells were prepared by breaking microscope slides under tissue paper. Adhesion to the different materials was measured using a blastospore concentration of 10^8 cells ml^{-1} incubated with discs for 4 h.

Influence of pH on adhesion to pPVC and uPVC

Blastospores were suspended to 10^8 blastospores ml^{-1} in PBS adjusted to pH values in the range 2 to 13. Rows of 5 discs of both pPVC and uPVC were exposed for 4 h to blastospores at each pH value. pH values in the PBS solutions were kept constant throughout the washing procedure.

Influence of electrolyte concentration on adhesion to pPVC and uPVC

Blastospores were suspended to 1 × 10^8 blastospores ml^{-1} in de-ionized water containing the electrolyte NaCl in the concentration range 0 to 100 mM. uPVC and pPVC discs in rows of 5 were exposed to blastospores at each NaCl concentration for 4 h. NaCl electrolyte concentrations were kept constant throughout the washing procedure.

Microelectrophoresis

Zeta potentials, a measure of the net charge on the surface of the blastospores, were measured in PBS at a range of pH values at room temperature with a Lazer Zee Meter 501 (PenKem), which uses the scattering of incident laser light to detect cells at

relatively low magnifications. The absolute electrophoretic mobilities can be derived directly from the velocities of the organisms in the applied electric field, the applied voltage, and the dimensions of the electrophoresis chamber (James, 1991). Electrophoretic mobilities were measured for blastospores suspended to a concentration of $\approx 1 \times 10^7$ blastospores ml^{-1} and converted into zeta potentials on the basis of the Helmholtz-Smoluchowski equation (Hiemenz, 1977).

Low-temperature scanning electron microscopy (LTSEM)

pPVC and uPVC discs were incubated with 1×10^8 blastospores ml^{-1} for 4 h before undergoing washing, fixation and crystal violet staining as under normal assay conditions. The discs were then rapidly frozen by being plunged into nitrogen slush and transferred to a Cambridge 200 SEM.

Once inside the microscope, ice on the surface of the discs was sublimed at -65°C until all visible ice crystals had disappeared. The discs were withdrawn into the prechamber and sputtercoated with gold. The specimen stub was returned to the cold stage set at minus 170°C and observed.

RESULTS

Adhesion assay

To determine the reproducibility of the adhesion assay, adhesion values were compared statistically by analysis of variance. Disc to disc (intrabatch) variation was quantified for both uPVC and pPVC by comparison of adhesion values derived from discs incubated with the same batch of *A. pullulans* blastospores. No significant disc to disc variation ($P > 0.05$) occurred for either material among the means of 5 rows of 5 discs incubated in the wells of a tissue culture plate. The individual mean and standard deviation values for percentage surface cover with blastospores among the five rows ranged between 32.3 ± 2.3 and 39.5 ± 2.7% for pPVC and between 2.5 ± 0.4 and 3.4 ± 1.4% for uPVC.

However, for both uPVC and pPVC significant interbatch variation ($P < 0.001$) occurred among mean adhesion values from 5 separate batches of blastospores. Individual mean and standard deviation values for percentage surface cover with blastospores among each of the 5 batches ranged between 31.5 ± 3.1 and 43.2 ± 3.0% for pPVC, and between 2.4 ± 0.4 and 15.9 ± 3.0% for uPVC. To eliminate this source of variation, each subsequent adhesion experiment was completed with blastospores from a single batch.

Kinetics of adhesion to pPVC and uPVC

The kinetics of adhesion of *A. pullulans* blastospores to pPVC and uPVC were examined by monitoring percentage surface cover of discs with adhered blastospores over a 10 h period (Fig. 1). Maximum adhesion to pPVC was 280% greater than that to uPVC at 4 h. Adhesion to uPVC rose quickly to 7% in 1 h and then more slowly to reach a plateau of 11.3% by 6 h. In contrast, the percentage of the pPVC disc surface covered with attached blastospores increased rapidly up to 3 h and reached a maximum of 43% surface cover after 4 h of incubation. Regular checks for blastospore germination on both uPVC and pPVC surfaces were made throughout the 10 h incubation period and no incidence of germination was observed. A second timecourse experiment investigated the possibility that the adhesion plateau observed on pPVC was an artefact of the settling process which occurred during incubation and that more blastospores could potentially attach. After a 6 h adhesion period, unbound spores were washed from discs and replaced with a fresh suspension of 10^8 blastospores ml^{-1} in PBS. Adhesion was then followed for an additional 6 h period. Replacement of the spore suspension resulted in no further increase in adhesion (data not shown), and the

percentage surface cover did not increase beyond a maximum of 43%, suggesting that saturation of binding sites on the pPVC had occurred. Since maximal adhesion on pPVC occurred after 4 h, this time was subsequently chosen as the incubation period.

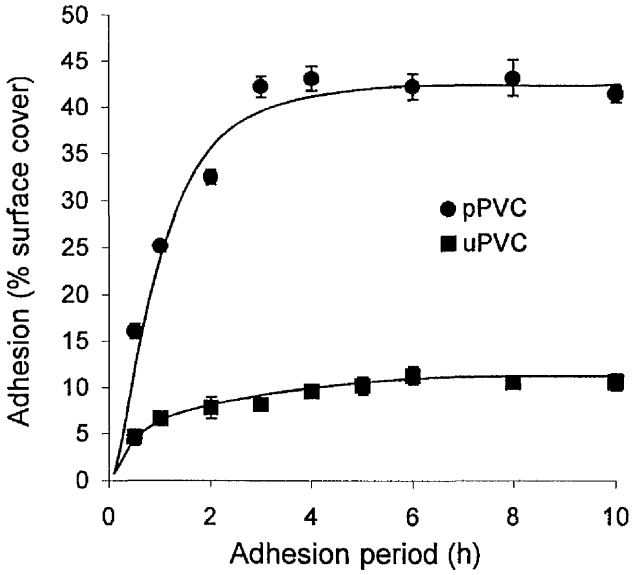


Fig. 1. Timecourse of adhesion of *A. pullulans* IMI70103 blastospores to uPVC and pPVC. Error bars show ±1 SE of the mean.

In order to determine the effect of increasing spore concentration on adhesion, percentage cover of pPVC and uPVC discs was determined after 4 h at spore concentrations in the range 5.2×10^6 to 3×10^8 blastospores ml^{-1} (Fig. 2). The binding isotherm for *A. pullulans* to pPVC showed that as the concentration of unbound spores increased to 7×10^7 blastospores ml^{-1}, adhesion to pPVC increased quickly to 30.6% surface cover, slowed over the concentration range 7×10^7 to 1.3×10^8 blastospores ml^{-1} and reached a maximum of 33.0% surface cover at a concentration of 1.8×10^8 blastospores ml^{-1}. Percentage cover of the pPVC surface with blastospores did not exceed 33% (1.1×10^5 blastospores mm^{-2}) in this experiment. Adhesion to uPVC increased rapidly to 5.8% surface cover in the concentration range 5.2×10^6 - 2.3×10^7 blastospores ml^{-1} and reached a plateau of 7.6% surface cover with blastospore concentrations of 1.0×10^8 ml^{-1} and above.

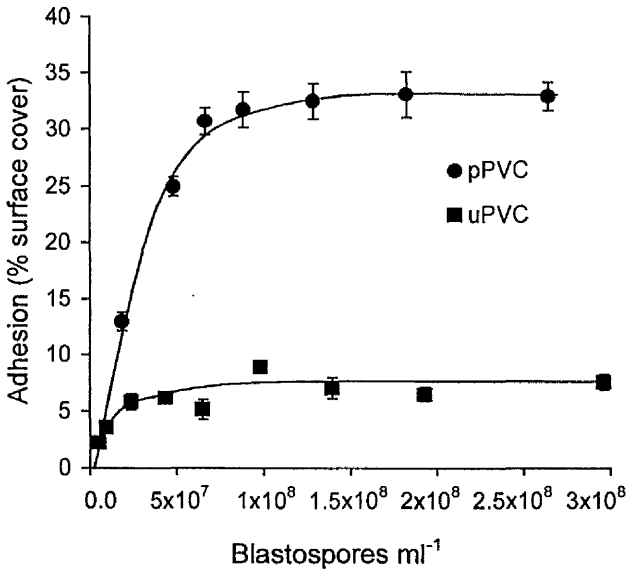


Fig. 2. Influence of blastospore concentration on adhesion of *A. pullulans* to uPVC and pPVC. Error bars show ±1 SE of the mean.

Influence of plasticizers on adhesion

To determine whether the increased adhesion to pPVC relative to uPVC was due to an attractive interaction between blastospores and the plasticizers, adhesion to uPVC was measured using blastospores suspended in a range of concentrations of a mixed dispersion of DOP and DOA (Fig. 3). Adhesion of *A. pullulans* blastospores to uPVC was increased by up to 308% by incorporation of the plasticizers into the suspension medium. Adhesion increased most rapidly within the plasticizer concentration range 0 to 15% of the undiluted dispersion, where percentage surface cover of the uPVC with blastospores increased from 6.2 to 20.7%. The rate of increase in adhesion slowed during the plasticizer concentration range 15 to 50%, reaching a plateau in adhesion of 25.4% surface cover with blastospores when the relative concentration of plasticizers was 50% of the undiluted dispersion. While adhesion to uPVC was strongly dependent on the concentration of dispersed plasticizers present in PBS, dissolved plasticizers remaining after removal of the dispersion did not influence the attachment of blastospores to the plastic (Fig. 3). Adhesion remained constant at 8% surface cover across the relative dissolved plasticizer concentration range of 0 to 100% of the undiluted solution.

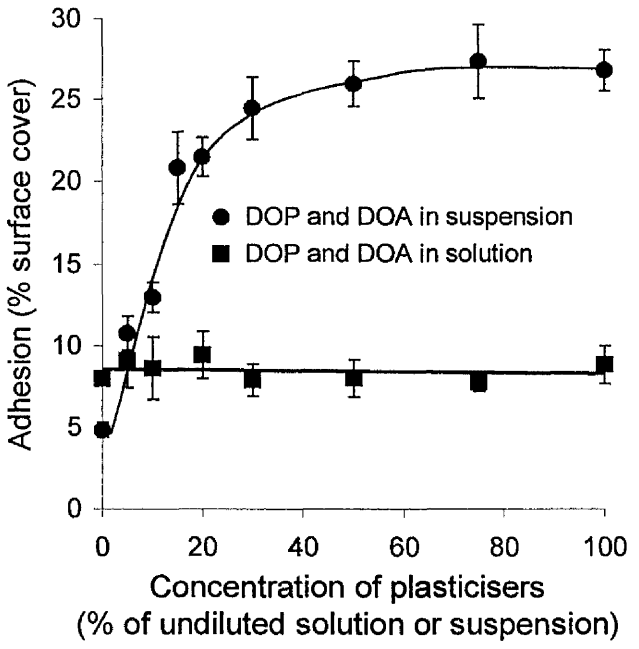


Fig. 3. Influence of the plasticizers DOP and DOA both as a suspension and dissolved in PBS on adhesion of *A. pullulans* blastospores to uPVC. Error bars show ±1 SE of the mean.

Substratum hydrophobicity

The importance of substratum hydrophobicity in blastospore adhesion was investigated (Fig. 4) by comparing levels of adhesion to a range of materials with different water contact angles. The lowest adhesion was to the relatively hydrophilic glass, where the percentage cover of the surface with blastospores did not exceed 1%. The highest adhesion levels of 41% surface cover were observed on pPVC. A clear relationship existed between increasing water contact angle of the surface and increased adhesion of blastospores for the majority of materials tested except pPVC. Adhesion to pPVC was approximately 660% higher than would be expected due to its hydrophobicity. In contrast, adhesion of blastospores to uPVC fitted the relationship between adhesion and hydrophobicity. Despite a relative reduction of 13° in the water contact angle of pPVC compared to that of uPVC, adhesion to pPVC was 163% greater than to uPVC.

Influence of pH and electrolyte concentration

To investigate whether incorporation of plasticizers into PVC influences electrostatic interactions between blastospores and the substratum, adhesion to both pPVC and uPVC was compared at a range of different pHs and electrolyte concentrations of the

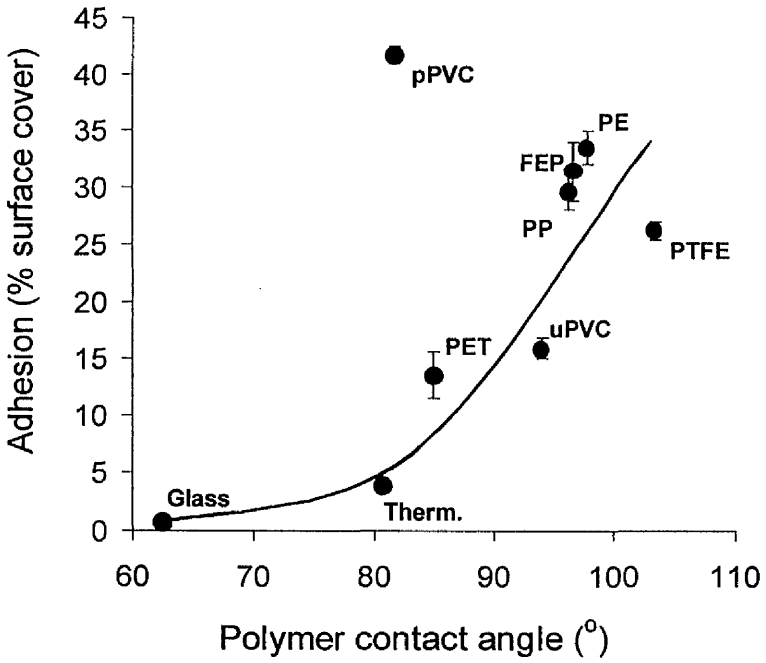


Fig. 4. Effect of increasing surface contact angle on adhesion of *A. pullulans IMI70103* to different substrata. Therm, Thermanox (tissue culture treated polyethylene tetraphthalate); PET, polyethylene tetraphthalate; PTFE, polytetrafluoroethylene; PP, polypropylene; FEP, fluoroethylene polypropylene; PE, polyethylene; uPVC, unplasticized polyvinyl chloride; pPVC, plasticized PVC. Error bars show ±1 SE of mean.

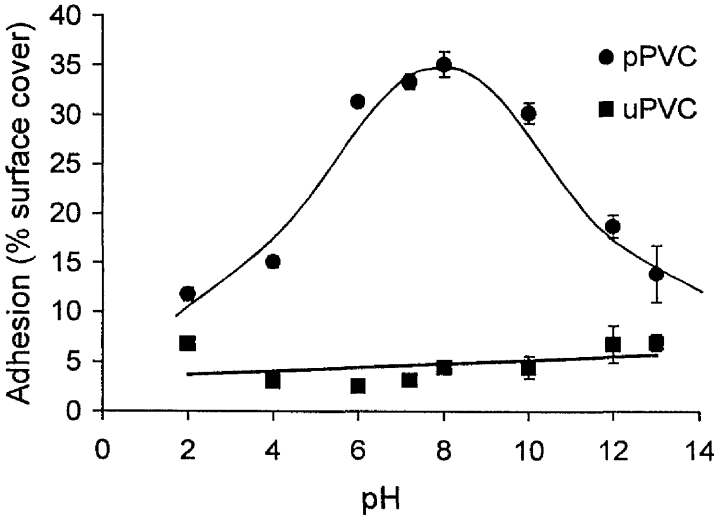


Fig. 5. Influence of suspension buffer pH on adhesion of *A. pullulans* blastospores to pPVC and uPVC. Error bars show ±1 SE of mean.

suspension buffer. Adhesion to pPVC was strongly influenced by pH (Fig. 5) with maximum adhesion occurring in the pH range 6 to 10. Percentage surface cover with blastospores rose from 12 to 35% as pH increased from 2 – 8, reducing to 14% surface cover at pH 13. In contrast, adhesion of blastospores to uPVC was unaffected by changing the pH of the suspension buffer.

Increasing molarities of NaCl were added to suspensions of blastospores in de-ionized water prior to incubation with pPVC discs (Fig. 6). Percentage cover of disc surfaces with attached blastospores increased from 6% with no electrolyte to a maximum of 30% across the optimal concentration range of 6 to 12 mM NaCl. A subsequent reduction in adhesion to 13% surface cover occurred as the electrolyte concentration was further increased to 100 mM. Adhesion to uPVC was unaffected by the electrolyte concentration within the suspension buffer.

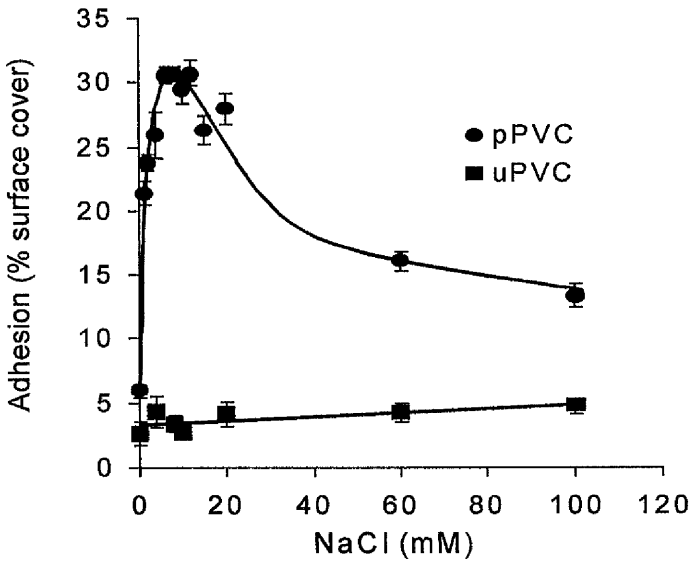


Fig. 6. Effect of electrolyte (NaCl) concentration on adhesion of *A. pullulans* IMI70103 to pPVC and uPVC. Error bars show ±1 SE of the mean.

Influence of pH on blastospore cell surface charge. To study the effect of pH on the cell surface electrostatic properties of *A. pullulans* blastospores, the zeta potentials of the blastospores were measured in PBS as a function of pH (Fig. 7). The blastospores demonstrated pH-dependent zeta potentials and possessed an isoelectric point within the pH range used, approximately at pH 5. Zeta potentials ranged from +13 mV (pH 2) to -13 mV (pH 12).

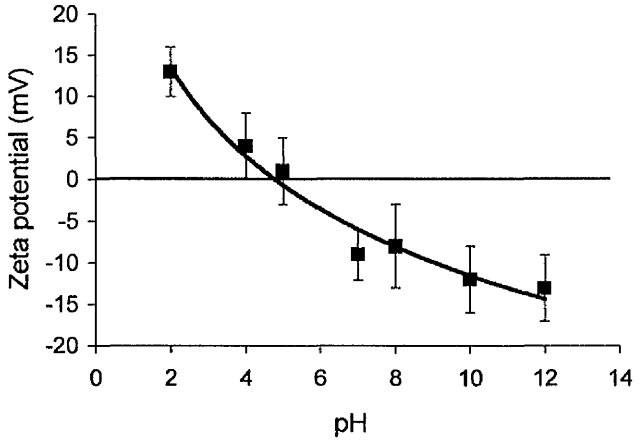
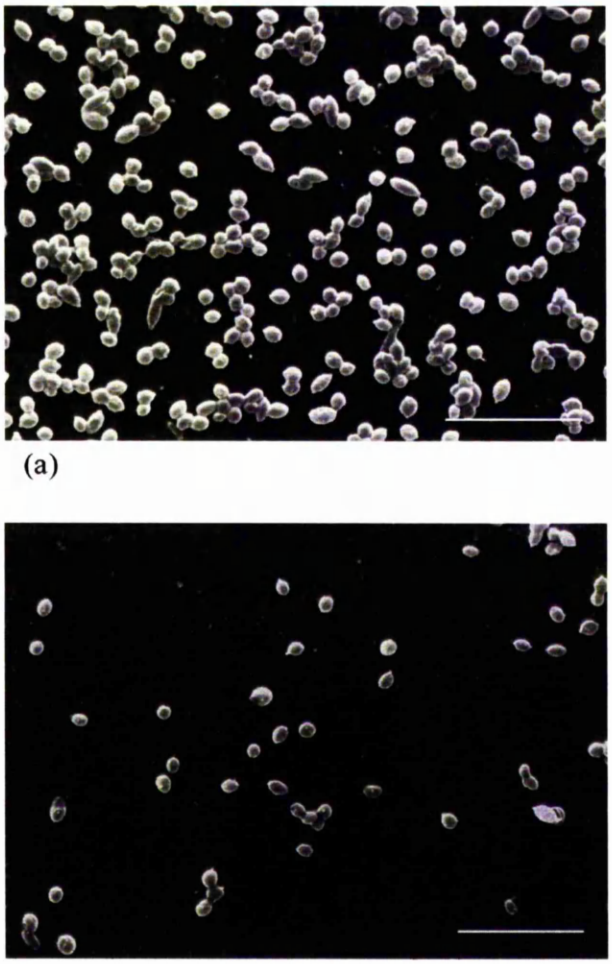


Fig. 7. Zeta potentials of *A. pullulans* IMI70103 blastospores in PBS as a function of pH. Data points represent averages for duplicate zeta potential measurements; Error bars show ± 1SD of mean.

LTSEM of pPVC and uPVC with attached blastospores. Blastospores were attached to uPVC and pPVC discs by incubation with 10^8 blastospores ml^{-1} for 4 h. Discs were examined under LTSEM subsequent to the normal washing, fixation, and staining procedures within the adhesion assay (Fig. 8). Blastospores were observed to be randomly dispersed on the surface of the discs and occurred as either single cells or aggregated into clumps. However, there was a far greater density of blastospores on the surface of the pPVC (Fig. 8a) than that of uPVC (Fig. 8b). The surfaces of both the uPVC and pPVC discs appeared smooth under LTSEM.



(a)

(b)

Fig. 8. Low temperature SEM of blastospores attached to (a) pPVC and (b) uPVC discs following incubation with 1 x 10^8 blastospores ml^{-1} for 4 h. Discs were washed, fixed and stained as normal for the adhesion assay. Bar = 20 μm.

DISCUSSION

The plasticizers DOP and DOA clearly enhance adhesion of *A. pullulans* blastospores to PVC and the physicochemical basis for this enhanced adhesion has been elucidated by using a rapid and quantitative adhesion assay. The plasticizers increased adhesion of blastospores to uPVC by a maximum of 308% when presented as a colloidal suspension (Fig 3.), indicating that there is an affinity of the blastospores for DOA and DOP. Equally high levels of adhesion occurred when blastospores were exposed to uPVC discs pretreated with plasticizer suspension for 1 h (data not shown). Thus the plasticizers are probably coating the uPVC surface resulting in an increase in adhesion of blastospores mediated by DOP and DOA in a concentration dependant manner. On the basis of these observations, subsequent experiments were designed to investigate the nature of the interaction of blastospores with both uPVC and pPVC.

Plasticizers can be utilized as a carbon source by *A. pullulans*. Consequently, DOA and DOP could increase adhesion indirectly by stimulating metabolic activity and the synthesis of adhesive cell surface structures. The ability of some fungi to attach to substrata is affected by their exposure to respiration inhibitors (Jones & Epstein, 1989; Sela-Buurlage *et al.*, 1991). However, 20 mM sodium azide, a mitochondrial respiration inhibitor, had no apparent effect on the adhesion of *A. pullulans* blastospores to pPVC but caused a 100% reduction in viability of the blastospores (see Appendix 4, p148). Therefore use of plasticizers as a carbon source by *A. pullulans* is unlikely to contribute to the increased levels of adhesion observed in the presence of plasticizers.

Plasticizers could also influence adhesion indirectly by leaching from the pPVC and dissolving in the liquid phase. Leached plasticizers could alter the physicochemical properties of the blastospore cell surface or of the suspension medium, for example by acting as surfactants. The ability of plasticizers to leach from pPVC into an aqueous environment is well established (Murase *et al.*, 1994; Wilson, 1995). However, dissolved plasticizers did not influence adhesion of *A. pullulans* to uPVC (Fig. 3). Furthermore, the water contact angle on uPVC, which would be greatly reduced in the presence of a surfactant, was not influenced by the incorporation of a mixed dispersion of DOP and DOA into the wetting fluid (see Appendix 3, p148). Therefore the plasticizers do not act as surfactants and the quantities of plasticizer that may leach from the surface of the pPVC are insufficient to cause the observed difference in adhesion to pPVC and uPVC. Thus it is likely that plasticizers enhance adhesion by

directly influencing physicochemical interactions, such as hydrophobic or electrostatic forces, between blastospores and the PVC substratum.

Hydrophobic interactions control adhesion of *A. pullulans* blastospores to the unplasticized polymers studied, including uPVC. This is evident from the strong positive correlation between increasing substratum hydrophobicity and adhesion observed among the range of polymers tested. Further, electrostatic interactions do not play a detectable role in the adhesion of *A. pullulans* blastospores to uPVC because adhesion was not influenced by either the pH or the electrolyte concentration of the suspension medium. The importance of substratum hydrophobicity in fungal adhesion to polymers is now well recognised. Increased substratum hydrophobicity has also been shown to correlate with increased adhesion of ungerminated conidia of plant pathogenic fungi (Sela-Buurlage *et al.*, 1991; Doss *et al.*, 1993; Terhune & Hoch, 1993) and of yeast cells of *C. albicans* (Klotz *et al.*, 1985; Hazen, 1989) to various substrata. However, attachment of blastospores to pPVC clearly did not fit the relationship between substratum hydrophobicity and adhesion, suggesting that electrostatic interactions may play an additional role in blastospore adhesion to pPVC.

Evidence for the involvement of electrostatic forces in adhesion to pPVC comes from the effect of pH on adhesion. pH influences the cell surface charge of blastospores (Fig. 7) and also exerts a major effect on their adhesion to pPVC, which was optimal at pH 8 and minimal at pH 2 and 13. pPVC containing the plasticizers DOP and DOA has been shown to have a net negative surface charge at pH 7.4 in PBS (Jones, 1997). Therefore reduced adhesion at pH values above 8 is presumably due to electrostatic repulsion between negatively charged blastospores and a negatively charged pPVC substratum. Support for this hypothesis comes from the observation that adhesion increased concurrently with a reduction in the negative zeta potential of the blastospores in the pH range 6 to 12. However, high levels of adhesion were also predicted at pH values in the range 2 to 5 due to electrostatic attraction between positively charged blastospores and the pPVC surface. The observed inhibition of adhesion at low pH values suggests that other factors controlling adhesion, such as the electrostatic charge of the substratum, are also influenced by the changes in pH of the suspension medium. Reduced adhesion at both high and low pH values has previously been described in the adhesion of bacteria to stainless steel (Stanley, 1983; Vanhaecke *et al.*, 1990) and was interpreted to be caused by changes in surface charge of both the bacterial cell and the substratum. For example, it is possible that surface charge may be altered by hydrolysis of the

plasticizers to their free acids at extremes of pH. Further, measurements of the zeta potential at the surface of capillary tubes have demonstrated that several polymers, including PVC, may acquire a net positive charge at low pH values (Schützner & Kenndler, 1992). In our study, it is possible that both the spores and the pPVC are protonated at low pH and that adhesion is inhibited due to electrostatic repulsion between the two positively charged surfaces.

Further evidence in support of electrostatic interactions between blastospores and pPVC is provided by the effect of the electrolyte concentration on adhesion. A major reduction in adhesion was observed when the blastospores were suspended in de-ionized water. This reduction was presumed to be due to electrostatic repulsion which is more pronounced in solutions of low ionic strength. Similar inhibition of adhesion at low ionic strength has previously been demonstrated among both bacteria (Stanley, 1983; Rönner *et al.*, 1990) and *C. albicans* (Jones & O'shea, 1994; Klotz *et al.*, 1995). Maximum adhesion to pPVC occurred at 0.01 M NaCl at which point electrostatic repulsion between the blastospore and the pPVC was presumed to be at a minimum. The subsequent decrease in adhesion at NaCl concentrations above 0.01 M may result from reduced electrostatic interaction caused by high concentrations of electrolyte. Depending on the characteristics of the adhesive interaction, electrolytes may inhibit adhesion by screening short range electrostatic attraction between oppositely charged groups (Fletcher, 1980), or by modifying the conformation of cell surface molecules involved in adhesion (Rutter & Abbott, 1978). Either process could be responsible for the observed reduction in adhesion of blastospores to pPVC, although further study would be required to understand in detail the exact nature of the electrostatic interaction.

The adhesion assay identified significant batch to batch variability in adhesion of *A. pullulans* blastospores to PVC. While a number of assays to quantify adhesion of fungal conidia to polymer surfaces have previously been described (Doss *et al.*, 1993; Braun & Howard, 1994; Mercure *et al.*, 1994), none have fully investigated the reproducibility of adhesion to the substrata tested. Interbatch variation has previously been determined in studies of bacterial adhesion to polymer surfaces (John *et al.*, 1995; Jones *et al.*, 1996), but this study is the first to highlight that similar variation can occur among levels of adhesion of fungal conidia to different substrata. The reasons for variation in adhesion levels between batches are poorly understood but could be due to slight fluctuations in environmental conditions during growth and development of the blastospores. In

practice, since adhesion levels of *A. pullulans* blastospores were consistent within batches, valid comparisons of adhesion data could be made within each batch.

The kinetics of adhesion of *A. pullulans* to uPVC and pPVC suggest that both of these surfaces contain a finite number of binding sites which become saturated at blastospore concentrations above 10^8 blastospores ml^{-1}. SEM observations of blastospores on pPVC and uPVC indicate that these sites are distributed evenly over the substratum. Adhesion data are frequently interpreted in terms of the number of sites which are available for a microorganism to attach to a surface (Cowan, 1995; Doyle, 1991). However, saturation of the surface with blastospores may also imply negative co-operativity, i.e. that the presence of attached blastospores reduces the probability of others attaching in their vicinity. Negative-negative charge interactions that would occur between pPVC and blastospores, and between blastospores and blastospores would be expected to create a condition of negative co-operative binding. Negative co-operativity of this nature has previously been shown to occur in the adhesion of *C. albicans* to PET cover slips (Klotz *et al.*, 1985).

In summary, we have demonstrated that incorporation of plasticizers into PVC enhances adhesion of blastospores of the deteriogenic fungus *A. pullulans* through an interaction that is mediated by electrostatic forces. In contrast, adhesion to uPVC is controlled principally by hydrophobic attraction. The implication of these results is that plasticizers may accelerate the biodeterioration processes occurring on pPVC by enhancing fungal adhesion. Such information should be taken into account in the design of novel PVC formulations that utilize surface chemistry to reduce microbial attachment.

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Chapter 4.

Expression of cytosolic GFP in *Aureobasidium pullulans* allows observation and quantification of the fungus on pPVC

ABSTRACT

Understanding growth and colonization processes of the deteriogenic fungus *Aureobasidium pullulans* on plasticized polyvinyl chloride (pPVC) may lead to new strategies for its control. *A. pullulans* was transformed to express a red-shifted, mutated form of the *Aequoria* green fluorescent protein (GFP) using the vector pTEFEGFP (Vanden Wymelenberg *et al.*, 1997. Biotechniques 23:686-690). All morphotypes of *A. pullulans*, including hyphae, blastospores and chlamydospores expressed cytosolic GFP and fluoresced brightly. When applied to the pPVC surface, the transformed strain Ap1 *gfp* was readily observed using epifluorescence microscopy and was amenable to quantification using image analysis. Yeast-phase Ap1 *gfp* blastospores were allowed to adhere to pPVC and were incubated on the substratum under malt extract broth or minimal salts medium supplemented with yeast extract. Under both media, a switch from yeast-phase to mycelial-phase growth was always observed on the substratum. Percentage surface cover of the pPVC substratum with Ap1 *gfp* hyphae under malt extract broth was monitored over time using image analysis. After a lag phase of 15 h, percentage surface cover increased rapidly from 7% to 70% between 15 and 30 h, slowing to reach 80% cover after 40 h. This study demonstrates that GFP expression, together with quantitative image analysis, provides a powerful method for studies of fungus-substratum relationships and colonization processes.

INTRODUCTION

The deuteromycete *Aureobasidium pullulans* is the principal organism causing defacement of plasticized pPVC in temperate and tropical climates (Hamilton *et al.*, 1993; Webb *et al.*, 2000). *A. pullulans* is a ubiquitous and extremely variable fungus, with morphotypes including blastospores, swollen cells, chlamydospores and hyphae (Cooke, 1959). Currently there are no studies of the morphology and development of *A. pullulans* on pPVC. An understanding of the growth of this fungus during its colonization and biodeterioration of pPVC will aid in the development of new strategies for its control.

However, it is not easily possible to follow and quantify in real-time the development of a microorganism growing on an opaque substratum. Conventional light microscopy is often prevented because the substratum blocks the passage of light through the microscope, or because sufficient contrast cannot be obtained without use of toxic dyes. With environmental samples, contaminating surface debris also hinders direct observation using light microscopy. Fluorescent dyes, such as 4'-6'-diamidino-2-phenylindole (DAPI) (Herbert, 1990) or calcofluor white (Scheu & Parkinson, 1994), allow microorganisms to be readily visualized on the substratum using epifluorescence microscopy, but are also toxic to the organism. In addition, neither technique allows monitoring of an individual species within a mixed microbial community.

Several direct and indirect techniques exist to detect or quantify individual fungal species on surfaces (For a review see Drahos, 1991). Indirect assessment of colony forming units (CFUs) washed from a substratum has been the predominant method, but requires long incubation times and cannot be used to study relationships of the organism with the substratum or with other microorganisms. Fluorescence *in-situ* hybridization (FISH) is a relatively recent technique that allows detection of individual species within surface attached microbial communities by the use of specific RNA or DNA probes labelled with fluorochromes (Amann *et al.*, 1990; McSweeney *et al.*, 1993). This technique has been successfully used to detect and quantify *A. pullulans* on leaf surfaces (Li *et al.*, 1997), but is labour-intensive, toxic to the organism, and is limited by permeabilization and uptake of the probe.

The green fluorescent protein (GFP) of the jellyfish *Aequoria victoria* is an ideal marker system for living cells on surfaces. GFP fluoresces green and requires only the presence of oxygen to form the chromaphore; i.e. no external substrates or co-factors are required

(Chalfie *et al.*, 1994). The GFP gene may be transferred to and expressed in a wide variety of organisms including plants (Sheen *et al.*, 1995), animals (Cheng *et al.*, 1996), bacteria (Chalfie *et al.*, 1994), and fungi (Cormack *et al.*, 1997; Gordon *et al.*, 2000). As GFP does not normally interfere with the growth of the host, it is extremely useful for non-disruptive studies of cell-substratum and cell-cell relationships at the single-cell level.

This study demonstrates that expression of GFP in *A. pullulans* can be used for the detection and quantification of the fungus on pPVC. This system allows rapid assessment of surface colonization and provides a method to compare the effect of different pPVC formulations on the growth of *A. pullulans* in real-time *in-situ*.

MATERIALS AND METHODS

Aureobasidium pullulans (de Bary) Arnaud

A. pullulans strain PRAFS8 was provided by Avecia Biocides, Manchester, United Kingdom, and was maintained on malt extract agar (Oxoid, Unipath Ltd, Basingstoke, United Kingdom). To produce blastospores, cultures were grown to mid log-phase in 80 ml malt extract broth (Oxoid) by incubation at 25°C for 18 h, with shaking at 200 rpm. *A. pullulans* blastospore suspensions were prepared in sterile citric acid buffer (pH 5). This buffer was prepared by mixing separate solutions of citric acid (5.3 g liter^{-1} deionized water) and Na_2HPO_4, (7.1 g liter^{-1} deionized water) to the appropriate pH. Blastospores were washed three times by centrifugation at 36 000 × g for 5 min and resuspended in buffer to an optical density at 540 nm of 1.0. For long-term storage, blastospores were frozen at -80°C in 20% (v/v) glycerol solution.

Transformation.

Expression vector pTEFEGFP (Vanden Wymelenberg *et al.*, 1997), containing a red-shifted mutant GFP cDNA (pEGFP-1; Clontech Laboratories UK Ltd., Basingstoke) downstream of an *A. pullulans* translation elongation factor promoter, was introduced into *A. pullulans* by co-transformation with pAN7-1, a vector conferring hygromycin resistance (Punt *et al.*, 1987). Protoplasts were prepared and transformed as previously described (Wang et al., 1988) with 10 µg of both pTEFEGFP and pAN7-1 and transformants selected on potato dextrose agar (Oxoid) containing 1M sorbitol and 100 µg ml^{-1} hygromycin B. Transformants were screened for GFP fluorescence using an Olympus BH-2 epifluorescence microscope equipped with a HBO 100-W mercury arc lamp and a fluorescein isothiocyanate filter set (Olympus, Hamburg, Germany). All transformants exhibiting fluorescence were sub-cultured onto malt extract agar (Oxoid) containing 100 µg ml^{-1} hygromycin B. Integration of plasmid DNA in two of the transformants (termed Ap1 *gfp* and Ap2 *gfp*) was confirmed by Southern analysis (Southern, 1975). For general observation on microscope slides, transformants were grown on thin-layer (10 ml) potato dextrose agar (PDA) plates. Sections of agar containing Ap1 *gfp* colonies were removed using a scalpel and transferred to a microscope slide.

Comparison of growth of *A. pullulans* wild-type and Ap1 *gfp* strains

The growth *A. pullulans* wild-type and Ap1 *gfp* strains was compared at various time intervals over a 42 hr time period in 50 ml malt extract broth on a rotary shaker (200

rpm) at 25°C. At each time interval, 1 ml samples were removed from each of three replicate culture flasks, diluted to an optical density at 540 nm < 1.0, and the OD_{540} recorded. The mean OD_{540} and standard deviation for 3 replicate cultures of *A. pullulans* and Ap1 *gfp* strains were calculated at each time point.

Growth of Ap1 *gfp* on the surface of pPVC

pPVC sheets, 0.5 mm thick, contained the following additives (parts per hundred resin): dioctyl phthalate plasticizer (Exxon Chemicals, Southampton, United Kingdom), 25; dioctyl adipate plasticizer (Exxon Chemicals), 25; Lankromark LN138 calcium/zinc stabilizer, (Akcros Chemicals, Burnley, United Kingdom), 2; Lankroflex ED63 epoxidized oleate ester (Akcros Chemicals), 3; and titanium dioxide pigment (Tioxide Europe, Grimsby, United Kingdom), 10. pPVC pieces, 1 cm × 1 cm were cut using a scalpel and sterilized by swabbing with alcohol.

To monitor growth of Ap1 *gfp* on pPVC, suspensions of Ap1 *gfp* blastospores were prepared as described above. In order to allow attachment of blastospores to the pPVC, pPVC pieces were placed in the base of a Petri-dish, covered with 15 ml blastospore suspension, and incubated at 25°C for 4 h without shaking. After this time, pPVC pieces were washed by dipping 5 times in sterile deionized H_2O and placed into the base of a fresh Petri-dish. pPVC pieces were then covered with 15 ml of either malt extract broth or mineral salts medium supplemented with yeast extract (MSYE), which contained the following (g l^{-1} deionized water): K_2HPO_4, 7; KH_2PO_4, 3; $MgSO_4.7H_2O$, 0.1; and $(NH_4)_2SO_4$, 1; yeast extract, 0.5. Petri dishes were then incubated at 25°C with gentle agitation at 30 rpm on a swirling-plate (Model SB1, Stuart Scientific, Redhill, United Kingdom). At various time intervals, inoculated pPVC pieces were removed and washed gently by dipping twice in sterile deionized water. Samples were then placed onto a microscope slide, covered gently with a cover-slip and examined directly under epifluorescence microscopy before being replaced under the liquid medium.

Microscopy and image analysis

Specimens were examined with a Leica DM RXA microscope (Meyer Instruments, Houston, TX) equipped for epifluorescence with a HBO 100-W mercury arc lamp and with a filter set for fluorescein isothiocyanate (FITC). Images were recorded with a cooled charged-coupled device (CCD) video camera (Model CH250, Photometrics Ltd., Tucson, Arizona, USA) controlled by IP Lab processing software (Signal Analytics Corporation, Vienna, Virginia, USA). Fluorescence images taken in the RGB colour

format were pseudocoloured by removal of the red and blue colour channels using Adobe Photoshop 5.0 software (Adobe Systems Inc., Uxbridge). Determination of percentage surface cover of the pPVC surface with Ap1 *gfp* hyphae using image analysis was conducted as follows. RGB images were converted to grayscale format using Image Tool software (University of Texas Health Science Centre in San Antonio, TX, USA) and a region of interest (ROI) was selected to encompass an area in clear focus. Threshold levels were then set to include fluorescent cells only and cells were outlined, filled and converted to a 1-bit (black and white) image. The percentage of the ROI covered with cells was then calculated automatically. In order to determine the percentage surface cover over time of pPVC pieces incubated under malt extract broth, 3 images were taken from each of 3 replicate pPVC pieces at each time interval. The mean and standard deviation of percentage surface cover for the three pPVC pieces was then calculated.

RESULTS AND DISCUSSION

Twenty putative co-transformants were identified by epifluorescence microscopy after 5 days incubation on hygromycin B-supplemented medium. Southern analysis with genomic DNA derived from the two brightest fluorescing transformants (termed Ap1 *gfp* and Ap2 *gfp*) and the parental strain confirmed integration of pTEFEGFP (see Appendix 5, p149). Both of these transformants showed very bright cytoplasmic fluorescence similar to that described in *A. pullulans* by Vanden Wymelenberg *et al.* (1997). Fluorescence was visible both at the level of the colony and of the individual cell. Epifluorescence microscopy showed that transformant Ap1 *gfp* showed the highest fluorescence levels and this transformant was used for subsequent studies. Fig.1 shows GFP expression in Ap1 *gfp* blastospores and hyphae growing in potato dextrose agar. Some of the fine structure of the *A. pullulans* cells can be distinguished with GFP fluorescence, with septa and prominent vacuoles clearly visible. Some variation in fluorescence intensity is seen between cells, but this might be caused by some cells lying outside of the plane of focus of the microscope.

The effect of incorporation of pTEFEGFP and expression of GFP on growth in *A. pullulans* was investigated. This was necessary because it is possible that cell growth rate may influence adhesion and colonization processes. This may be caused, for example, by alterations of cell surface hydrophobicity, which have been shown to occur according to growth phase in bacteria (Van Loosedrecht *et al.*, 1987). In order to compare growth of *A. pullulans* wild type and Ap1 *gfp* strains, the optical density of both strains growing in malt extract broth was monitored over a 42 h period (Fig. 2). During this period, each grew solely as budding yeast-phase blastospores. Expression of GFP did not influence growth of *A. pullulans* in liquid culture. For both strains, the optical density increased exponentially from 4 – 16 h, entering stationary phase at approximately 20 h and reaching a plateau at 38 h. The mean doubling time for both strains during exponential growth was 2 h 10 min.

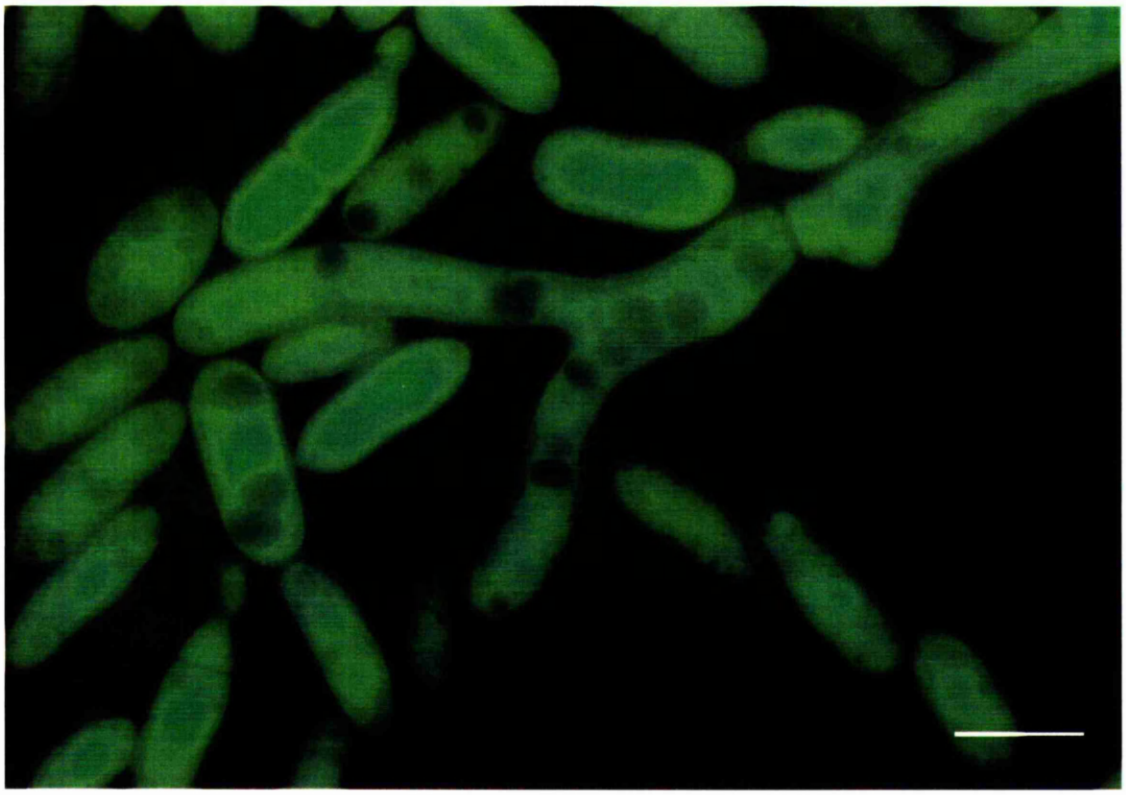


Fig. 1. Cytosolic GFP expression in Ap1 *gfp* hyphae and blastospores. Cells were photographed after 2 days growth in a thin layer of PDA. Bar = 10 μm

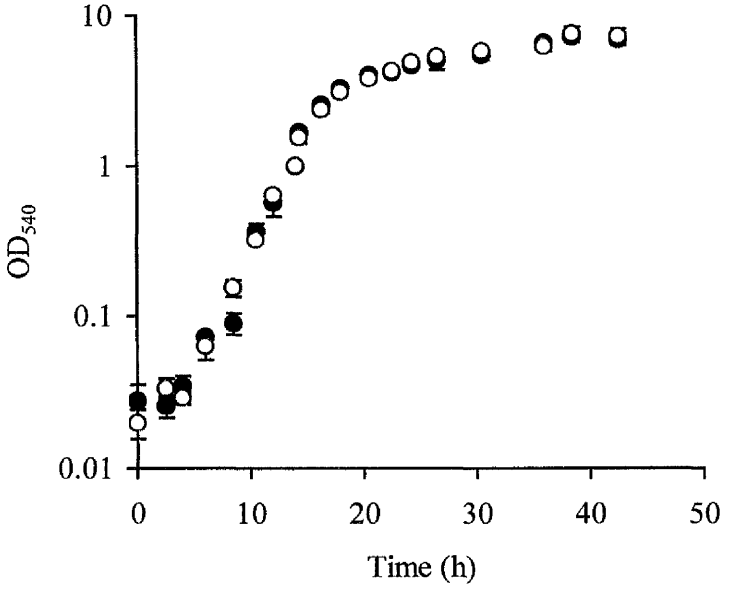


Fig. 2. Growth of *A. pullulans* wild type (●) and Ap1 *gfp* (○) strains in malt extract broth at 25°C.

GFP-labelled *A. pullulans* cells were readily observed on the surface of pPVC using epifluorescence microscopy. Growth of Ap1 *gfp* on pPVC under MMYE medium is shown in Fig. 3. MMYE medium was used because it contains very low quantities of carbon nutrients (0.5 g l^{-1} yeast extract) and therefore more closely represents the oligotrophic conditions that are likely to occur on pPVC exposed in the environment. Fig. 3a shows blastospores immediately after adhesion to the pPVC. Budding cells observed at this stage are probably not multiplying, but are a result of harvesting and carry-over of budding cells from mid-exponential phase growth in malt-extract broth. After 12 h, blastospores were observed to have swollen and become segmented into two or three cells through the establishment of septa (Fig. 3b). A switch from yeast-phase to mycelial-phase growth was subsequently observed on the substratum. Further division resulted in the development of hyphae after 24 h (Fig. 3c) and larger hyphal colonies after 48 h (Fig. 3d). Thus GFP fluorescence can be readily used to observe growth and development of *A. pullulans* in real-time on pPVC.

Growth of GFP-labelled Ap1 *gfp* cells was also amenable to quantification under image analysis. Percent coverage of the pPVC substratum with Ap1 *gfp* hyphae over time was quantified during growth under malt extract broth (Fig. 4.). Unlike in MMYE medium, discrete *A. pullulans* colonies were not observed on pPVC under malt extract broth. During the first 10 h incubation under malt extract broth, blastospores began to extend

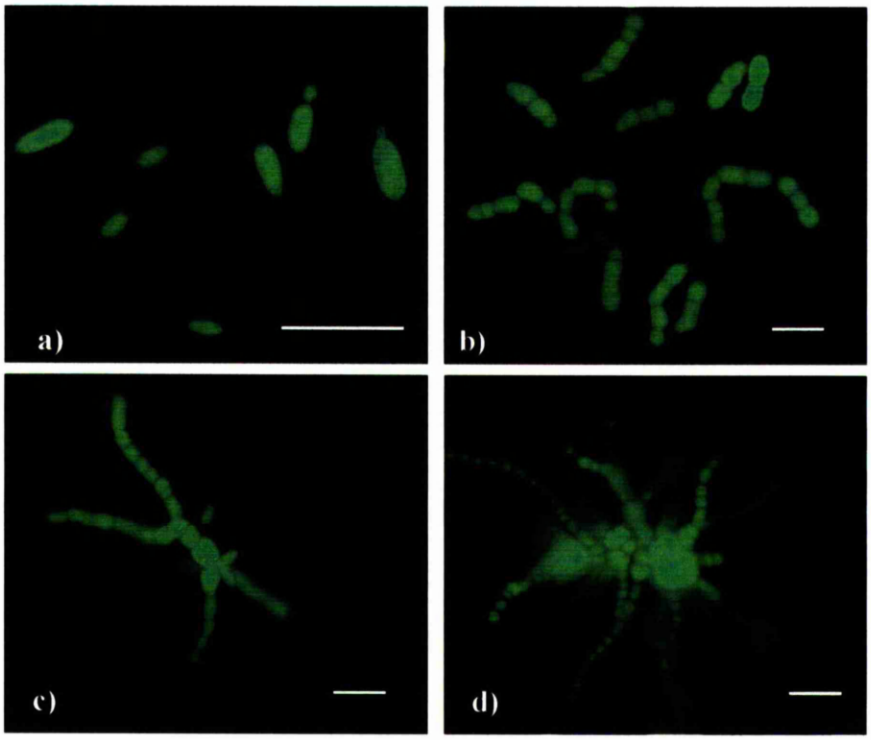
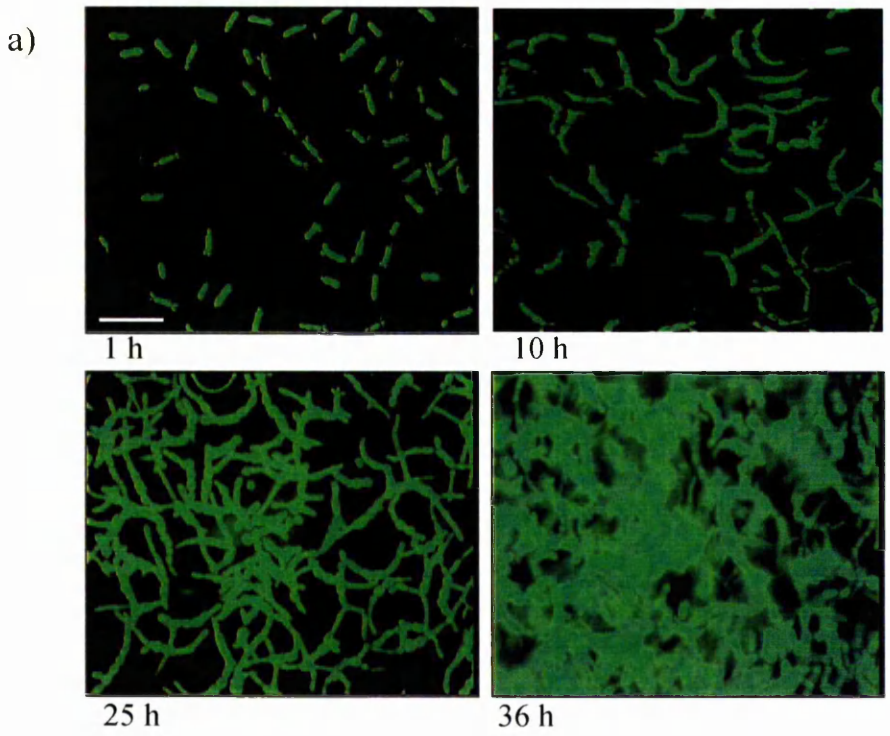


Fig. 3. Time-course of development of *A. pullulans* colonies on pPVC under minimal medium liquid supplemented with yeast extract (MMYE): a) 0 h, b) 12 h, c) 24 h, d) 48 h. Bar = 10 μm.

into hyphae (Fig. 4a) but little increase in percentage cover occurred. Between 15 and 30 h, percentage cover of the surface with hyphae increased rapidly from 7% to 70%, slowing to reach 80% surface cover after 40 h (Fig. 4b). Thus growth of Ap1 *gfp* on the pPVC substratum appeared to exhibit lag and exponential phases similar to those generally observed with growth of microorganisms in liquid batch culture. However, the reduced growth rate between 30 and 40 h may not be caused by cells entering stationary phase, but may be an artefact resulting from the vertical stacking of hyphae as the surface becomes saturated with the fungus. Vertical stacking of cells is a general problem with quantitative image analysis on surfaces (Li *et al.*, 1997). However, while multi-layering of cells became a problem during vigorous growth under malt-extract broth, multi-layered fungal biofilms would rarely occur on pPVC substrata exposed *in-situ* under nutrient limited conditions. The approximate mean doubling time of percent coverage of the pPVC with *A. pullulans* hyphae was 7 h and 20 min. This is a much lower doubling rate than that observed during batch culture (2 h 10 min). This difference in growth rate may be caused by the different culture conditions used for *A. pullulans* biofilm development and batch culture growth (stirred Petri-dish and shake-flask culture respectively). For example, shake-flask culture has greater levels of agitation and may provide higher oxygen levels to cells. Other possible causes of the reduced growth rate during surface colonization may include reduced cell-surface area available for nutrient uptake caused by surface attachment, and physiological or metabolic differences between yeast-phase and mycelial-phase *A. pullulans* cells.

In summary, GFP expression in *A. pullulans*, in combination with the accuracy and versatility offered by image analysis, provides an ideal method to study the colonization of surfaces by this organism. This technique will be useful for studies of the effect of different pPVC formulations on growth of *A. pullulans*, and therefore may aid in the development of surfaces that prevent or reduce microbial colonization. Further studies will also investigate the effect of different biocides incorporated into pPVC on GFP fluorescence and colonization of *A. pullulans*. It may also be possible to apply GFP tagged strains of *A. pullulans* to pPVC exposed *in-situ* to monitor growth and inter-species interactions of *A. pullulans* in the environment.



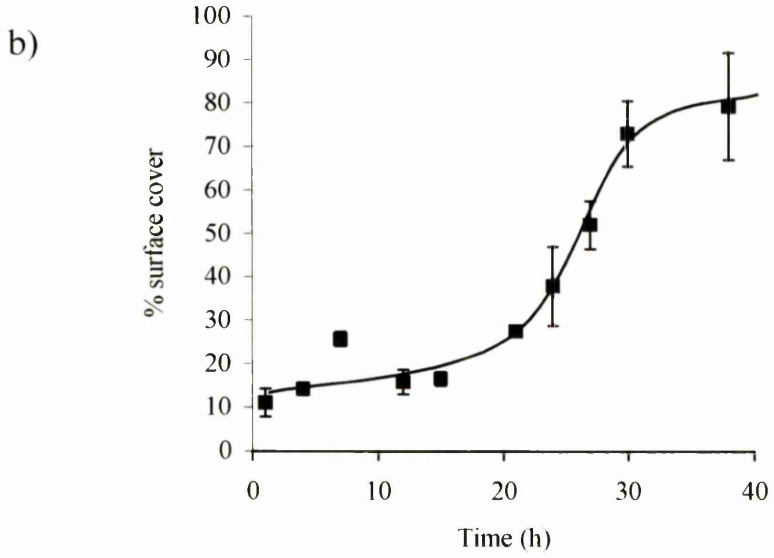


Fig. 4. Timecourse of growth of Ap1 *gfp* on pPVC under malt extract broth. a) Fluorescence microscopy of Ap1 *gfp* growth on the surface of pPVC at various time intervals; Bar = 10 µm b) percentage surface cover of the pPVC with time as determined using image analysis.

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Chapter 5.

GFP as a novel indicator of antimicrobial susceptibility in Aureobasidium pullulans.

Submitted to *Applied and Environmental Microbiology*

ABSTRACT

Currently there is no method available that allows non-invasive and real-time monitoring of fungal susceptibility to antimicrobial compounds. The green fluorescent protein (GFP) of the jellyfish *Aequoria victoria* was tested as a potential reporter molecule for this purpose. *Aureobasidium pullulans* was transformed to express cytosolic GFP using the vector pTEFEGFP (Vanden Wymelenberg *et al.*, 1997. Biotechniques 23:686-690). The transformed strain Ap1 *gfp* showed bright fluorescence that was amenable to quantification using fluorescence spectrophotometry. Fluorescence levels in Ap1 *gfp* blastospore suspensions were directly proportional to the number of viable cells determined by CFU plate counts ($R^2 > 0.99$). The relationship between cell viability and GFP fluorescence was investigated by adding a range of concentrations of each of the biocides sodium hypochlorite and 2-*n*-octylisothiazolin-3-one (OIT) to suspensions of Ap1 *gfp* blastospores (pH 5 buffer). These biocides each caused a rapid (< 25 min) loss of fluorescence of greater than 90% when used at concentrations of 150 $\mu g\ ml^{-1}$ available chlorine and 500 $\mu g\ ml^{-1}$ respectively. Further, loss of GFP fluorescence from *A. pullulans* cells was highly correlated with a decrease in the number of viable cells ($R^2 > 0.92$). Loss of GFP fluorescence and cell viability was highly dependant on external pH; maximum losses of fluorescence and viability occurred at pH 4 while reduction of GFP fluorescence was absent at pH 8.0 and was associated with a lower reduction in viability. This technique allowed the relative antimicrobial properties of a range of biocides to be compared using simple fluorescence measurements and should have broad applications in testing the susceptibility of *A. pullulans* and other fungal species to antimicrobial compounds.

INTRODUCTION

Since the cloning of wild-type GFP from the jellyfish *Aequoria victoria* (Prasher *et al.*, 1992), expression of GFP has been demonstrated in numerous organisms including plants (Sheen *et al.*, 1995), animals (Cheng *et al.*, 1996), bacteria (Chalfie *et al.*, 1994), yeasts (Cormack *et al.*, 1997), and filamentous fungi (Vanden Wymelenberg *et al.*, 1997; Gordon *et al.*, 2000). Most applications of GFP have been as a passive label of gene expression and protein localization (for a review, see Tsien (1998)). However, GFP and selected mutants are now increasingly used as active sensors of physiological events within cells. In this role, GFP fluorescence is influenced post-translationally by its chemical environment. For example, the pH-sensitivity of GFP has recently been exploited to measure intracellular (Kneen *et al.*, 1998; Llopis *et al.*, 1998; Robey *et al.*, 1998) and organellar (Llopis *et al.*, 1998) pH, and GFP-based systems have been developed to monitor intracellular calcium (Miyawaki *et al.*, 1997), microviscosity (Swaminathan *et al.*, 1997) and protease activity (Heim & Tsien, 1996).

One application of GFP that has not been explored in fungi is its use as an indicator of antimicrobial susceptibility. GFP has several properties that are desirable for this purpose, including simplicity and versatility for *in-vitro* use (Cubitt *et al.*, 1995). GFP is intrinsically fluorescent, requiring no co-factors or exogenous substrates. Problems related to cell permeabilization and uptake or retention of product are thus avoided (Chalfie *et al.*, 1994; Cubitt *et al.*, 1995). GFP fluorescence also has the advantage that it can be quantitated *in-situ* using a variety of techniques including fluorescence microscopy, (Vanden Wymelenberg *et al.*, 1997), flow cytometry (Dhandayuthapani *et al.*, 1995), and fluorimetry (Casey & Nguyen, 1995).

The ability to rapidly assess viability is important in the evaluation of susceptibility to antimicrobial compounds. Plate count methods are often used for this purpose, but are labour intensive and require long incubation times. Fluorescence-based assays of cellular viability, such as those based on fluorescein (Yang *et al.*, 1995) or tetrazolium salt derivatives (Rodriguez *et al.*, 1992), offer greater sensitivity and ease of use. However, most of these assays rely on the ability of cells to take up or metabolize extracellular fluorogenic compounds, and therefore may be limited by permeability of the cell membrane. In bacteria, bioluminescence using luciferase reporter genes provides a sensitive, non-invasive marker of cell viability (Stewart & Williams, 1992).

Bioluminescence can be measured *in-situ*, allowing measurement of cell viability for both planktonic (Duncan *et al.*, 1994) and surface attached bacteria (Kerr *et al.*, 1999).

In fungi there are no reports of the use of real-time, non-invasive reporters of cellular viability in the presence of antimicrobial compounds. Such a technique would have broad applications in environmental, industrial and medical mycology. We are interested in monitoring cell viability in *A. pullulans* because it is the predominant organism causing defacement and biodeterioration of plasticized poly(vinylchloride) (pPVC) (Hamilton, 1983; Webb *et al.*, 2000). The ability to rapidly assess viability of *A. pullulans* on pPVC is important in the evaluation of biocides that provide protection against biodeterioration of pPVC. The data presented here demonstrate a strong correlation between GFP fluorescence and cell viability in *A. pullulans* and suggest that this technique has considerable potential for the rapid and real-time evaluation of fungal susceptibility to antimicrobial compounds.

MATERIALS AND METHODS

Aureobasidium pullulans (de Bary) Arnaud

A. pullulans strain PRAFS8 was provided by Avecia Biocides, Manchester, United Kingdom, and was maintained on malt extract agar (Oxoid, Unipath Ltd, Basingstoke, United Kingdom). To produce blastospores, cultures were grown to mid log-phase in 80 ml malt extract broth (Oxoid) by incubation at 25°C for 18 h, with shaking at 200 rpm. *A. pullulans* blastospore suspensions were prepared in citric acid buffer (pH 5). This buffer was prepared by mixing separate solutions of citric acid (5.3 g $liter^{-1}$ deionized water) and Na_2HPO_4, (7.1 g $liter^{-1}$ deionized water) to the appropriate pH. Blastospores were washed three times by centrifugation at 36 000 × g for 5 min and resuspended in buffer to an optical density at 540 nm of 1.0. For long term storage, blastospores were frozen at -80°C in 20% (v/v) glycerol solution.

Transformation

Expression vector pTEFEGFP (Vanden Wymelenberg *et al.*, 1997), containing a red-shifted mutant GFP cDNA (pEGFP-1; Clontech) downstream of an *A. pullulans* translation elongation factor promoter, was introduced into *A. pullulans* by co-transformation with pAN7-1, a vector conferring hygromycin resistance (Punt *et al.*, 1987). Protoplasts were prepared and transformed as previously described (Wang *et al.*, 1988) with 10 μg of both pTEFEGFP and pAN7-1 and transformants selected on potato dextrose agar (Oxoid) containing 1M sorbitol and 100 μg ml^{-1} hygromycin B. Transformants were screened for GFP fluorescence using an Olympus BH-2 epifluorescence microscope equipped with a HBO 100-W mercury arc lamp and a fluorescein isothiocyanate filter set (Olympus). All transformants exhibiting fluorescence were subcultured onto malt extract agar (Oxoid) containing 100 μg ml^{-1} hygromycin B. Integration of plasmid DNA in transformants was confirmed by Southern analysis (Southern, 1975).

Measurement of GFP fluorescence

Suspensions of transformed *A. pullulans* blastospores were prepared as described above. Aliquots (1 ml) of blastospores were transferred to cuvettes and GFP fluorescence was quantified using a Hitachi F2000 fluorescence spectrophotometer with excitation at 485 nm and emission at 510 nm. Standard curves of optical density and viable cell number versus fluorescence were prepared by making serial dilutions of transformed blastospores in citrate-phosphate buffer (pH 5). Untransformed *A. pullulans*

blastospores were used as a control. Viable cells were enumerated by plating serial dilutions onto malt extract agar and incubating at 25°C for 3 days. To investigate the reproducibility (inter-batch variation) of GFP fluorescence levels, fluorescence measurements were made from blastospore suspensions prepared from 5 separate cultures.

Influence of biocides on GFP fluorescence and cell viability

The following biocides were obtained from Avecia biocides, Manchester, United Kingdom: 2-n-Octyl-4-isothiazolin-3-one (OIT); 2,3,5,6-tetrachloro-4-(methylsulphonyl)pyridine (TCMP); 10,10'-oxybisphenoxyarsine (OBPA); N-(trichloromethylthio)phthalimide (NCMP) and n-butyl-1,2-benzisothiazolin-3-one (BBIT). Stock solutions of these biocides were prepared in dimethyl sulfoxide (DMSO), so that the final concentration of DMSO added to blastospore suspensions was 2% (v/v). Sodium hypochlorite was obtained from BDH (Darmstadt, Germany) and was added directly to Ap1 *gfp* cells. Varying concentrations of the biocides OIT and sodium hypochlorite were added to 30 ml aliquots of blastospores in 50 ml centrifuge tubes (Falcon). Fluorescence measurements from three replicate tubes were made at various intervals for each biocide concentration and tubes were shaken throughout using a rotating mixer set at 30 rpm (Model SB1, Stuart Scientific, Redhill, United Kingdom). To monitor viable cell numbers during biocide treatment, 10 ml aliquots of blastospore suspensions were treated with either 100 $\mu g\ ml^{-1}$ OIT or 75 $\mu g\ ml^{-1}$ sodium hypochlorite for different time periods. At specific time points, fluorescence measurements were made from each tube and cells were immediately washed three times in citrate-phosphate buffer (pH 5) by centrifuging at 3 600 × g prior to plating on malt extract agar for enumeration. With sodium hypochlorite, sodium thiosulphite neutralizer solution was added to suspensions to a final concentration of 1% (w/v) before washing. These experiments were replicated on at least two separate occasions. The influence of an additional range of industrial biocides on GFP fluorescence and cell viability was determined at the working concentration at which they are normally incorporated within pPVC. These concentrations were ($\mu g\ ml^{-1}$): TCMP, 50; OIT, 500; BBIT, 750; OBPA, 50; NCMP 500. For each biocide GFP fluorescence was monitored over a period of 4 h, after which time cells were washed 3 times as previously described and plated on malt extract agar for enumeration.

Influence of external pH on GFP fluorescence and cell viability

The biocide OIT was used to investigate the influence of external pH on loss of GFP

fluorescence and viable cell numbers. Blastospore suspensions were prepared in citrate-phosphate buffer adjusted to pH values in the range 4 - 8. Aliquots (10 ml) of cells were treated with 100 μg ml^{-1} OIT for 1 hr. After this time fluorescence measurements were made from each tube and cells were immediately washed three times in buffer at the appropriate pH by centrifuging at 3 600 × g for 5 min. Numbers of viable cells remaining at each pH value were then determined by plating on malt extract agar. GFP fluorescence and CFU counts were compared relative to controls without added OIT at each pH value.

RESULTS

Transformation and colony screening

After incubation for 5 days, 20 putative transformants growing on hygromycin B-supplemented medium were identified by epifluorescence microscopy. Southern analysis with genomic DNA derived from two of the transformants (termed Ap1 *gfp* and Ap2 *gfp*) and the parental strain confirmed integration of pTEFEGFP (see Appendix 5, p149). Both of these transformants showed very bright cytoplasmic fluorescence similar to that described by Vanden Wymelenberg *et al.* (1997). Fluorescence spectrophotometry showed that transformant Ap1 *gfp* showed the highest fluorescence levels and this transformant was selected for biocide susceptibility studies.

GFP fluorescence

GFP fluorescence in Ap1 *gfp* blastospores was linear with respect to both optical density (Fig. 1a) and viable cell number (Fig. 1b) ($R^2 > 0.99$). Untransformed *A. pullulans* showed slight background fluorescence at high cell densities (Fig. 1a), probably due to scattering of incident light. Significant interbatch variation ($P < 0.001$) occurred among mean fluorescence values from five separate cultures of blastospores. The individual mean and standard deviation for fluorescence values among the five cultures ranged between 127 ± 1 and 197 ± 5 relative fluorescence units. In order to eliminate this source of variation, each subsequent experiment was completed from a single batch of blastospores.

Influence of biocides on GFP fluorescence

Both of the biocides OIT and sodium hypochlorite caused rapid losses of GFP fluorescence from Ap1 *gfp* cells in a concentration-dependant manner (Fig. 2). In the presence of 500 μg ml^{-1} OIT, fluorescence levels fell by 82% in 30 s, then reduced more slowly to reach a plateau at 95% loss of fluorescence by 25 min (Fig. 2a). Interestingly, the influence of 100 μg ml^{-1} OIT on fluorescence appeared to be biphasic. During the first 17 minutes of incubation, fluorescence levels fell by 30% at a rate similar to that observed using 50 μg ml^{-1} OIT. However, fluorescence levels then fell by a further 40% at an increased rate between 17 min and 1 h of incubation. This biphasic reduction in fluorescence was consistently observed in over 5 independent experiments. In comparison with OIT, incubation with sodium hypochlorite caused similar reductions in fluorescence in the range 15% (25 μg ml^{-1} available chlorine) to 90% (150 μg ml^{-1} available chlorine) after 35 min (Fig. 2b). Measurements of the effects of both biocides on GFP fluorescence were repeated on at least 3 separate occasions with similar results.

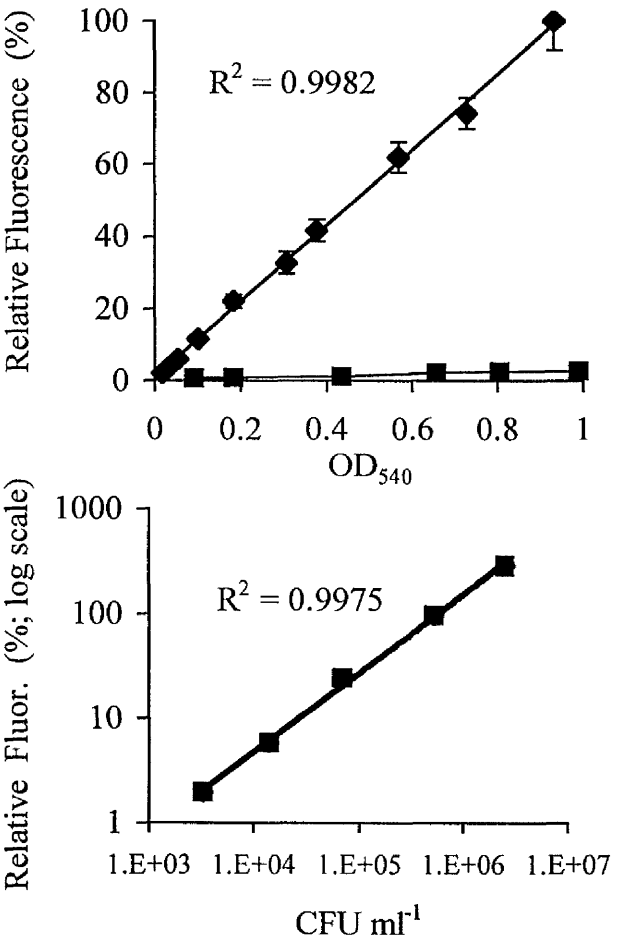

Fig. 1. GFP fluorescence in Ap1 *gfp* blastospores. a) Correlation between optical density at 540 nm and fluorescence for Ap1 *gfp* (◆) and the parental strain (■); b) correlation between viable cell numbers and GFP fluorescence.

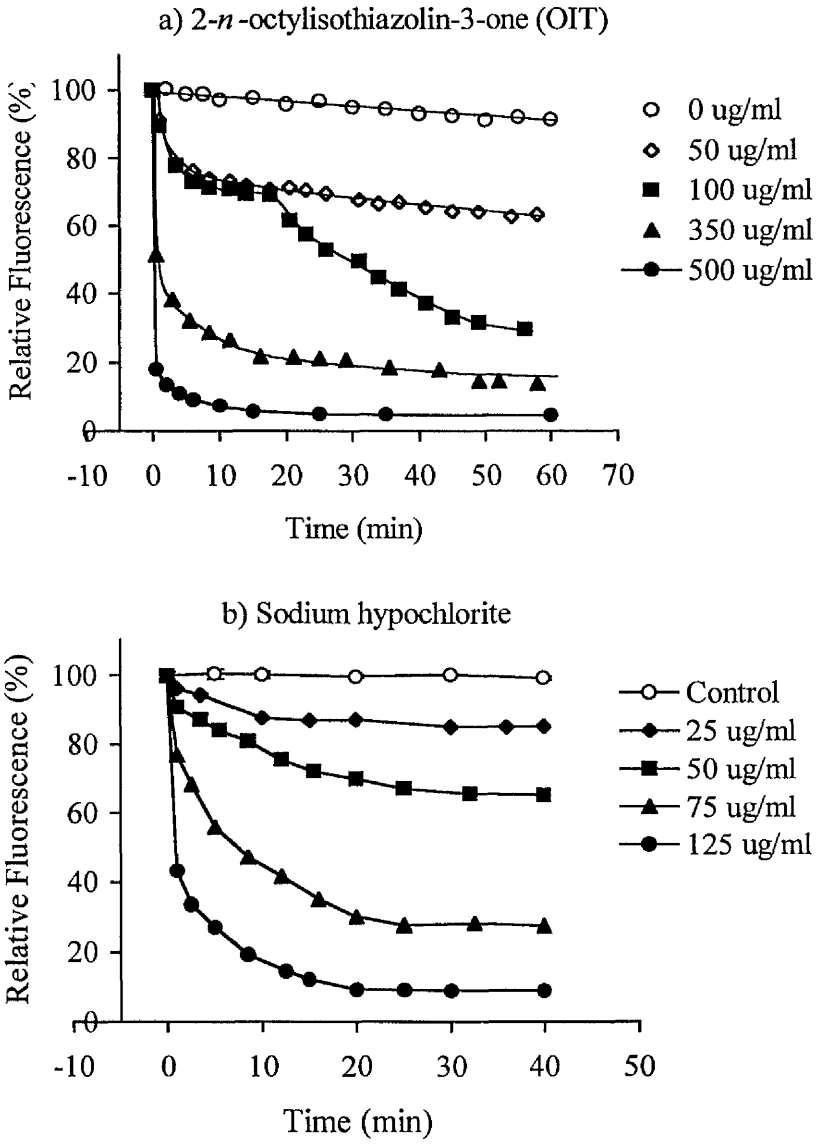

Fig. 2. Influence of the biocides OIT and sodium hypochlorite on GFP fluorescence in Ap1 *gfp* blastospores. a) OIT at a concentration of 0(○), 50 (◇), 100 (■), 350 (▲) and 500 (●) μg ml^{-1}; b) sodium hypochlorite at a concentration of 0 (○), 25 (◆), 50(■), 75(▲) and 125 (●) μg ml^{-1} available chlorine.

Correlation between GFP fluorescence and cell viability

To determine whether loss of GFP fluorescence correlated with a reduction in the number of viable cells, fluorescence measurements and CFU counts were made at intervals after the addition of each of the biocides OIT and sodium hypochlorite at 100 μg ml^{-1} and 75 μg ml^{-1} available chlorine respectively (Fig. 3). With OIT, logarithmic decreases in the number of viable cells paralleled the loss of GFP fluorescence and CFU counts fell from 3.5×10^6 to 6.3×10^2 CFU ml^{-1} in 1 h (Fig. 3a). Sodium hypochlorite caused a rapid decrease in viability from 5×10^6 to 1.8×10^4 CFU ml^{-1} by 30 s, and this mirrored the large reduction in GFP fluorescence of 38% which also occurred within 30 s (Fig. 3b). With sodium hypochlorite, decreasing CFU counts reached a plateau at 9.2×10^2 CFU ml^{-1} after 10 min, while GFP fluorescence continued to decrease slowly after this time and reached a plateau of 11% relative fluorescence after 20 min (Fig. 3b). The data for both biocides showed a strong linear correlation ($R^2 = 0.93$) between loss of GFP fluorescence and logarithmic decreases in CFU viable counts (Fig. 3c). Measurements of GFP fluorescence loss and cell viability were made on two separate occasions for each biocide with similar results.

Influence of external pH on fluorescence and cell viability

Both loss of fluorescence and cell viability in the presence of OIT were highly dependant on the pH of the suspension buffer (Fig. 4). Loss of GFP fluorescence was greatest under acidic conditions. The maximum reduction of 73% occurred at pH 4, while no significant loss of fluorescence ($P < 0.001$) occurred under alkali conditions at pH 8. Fluorescence levels remaining after treatment with OIT for 1 h increased in a linear manner between pH 4 and pH 8. Loss of cell viability was also greatest at acidic pH values, with >99.98% loss of viability at pH 4, while 39% of cells remained viable at pH 8. A linear log-log relationship occurred between external pH and viable cell numbers remaining after incubation with OIT for 1 h.

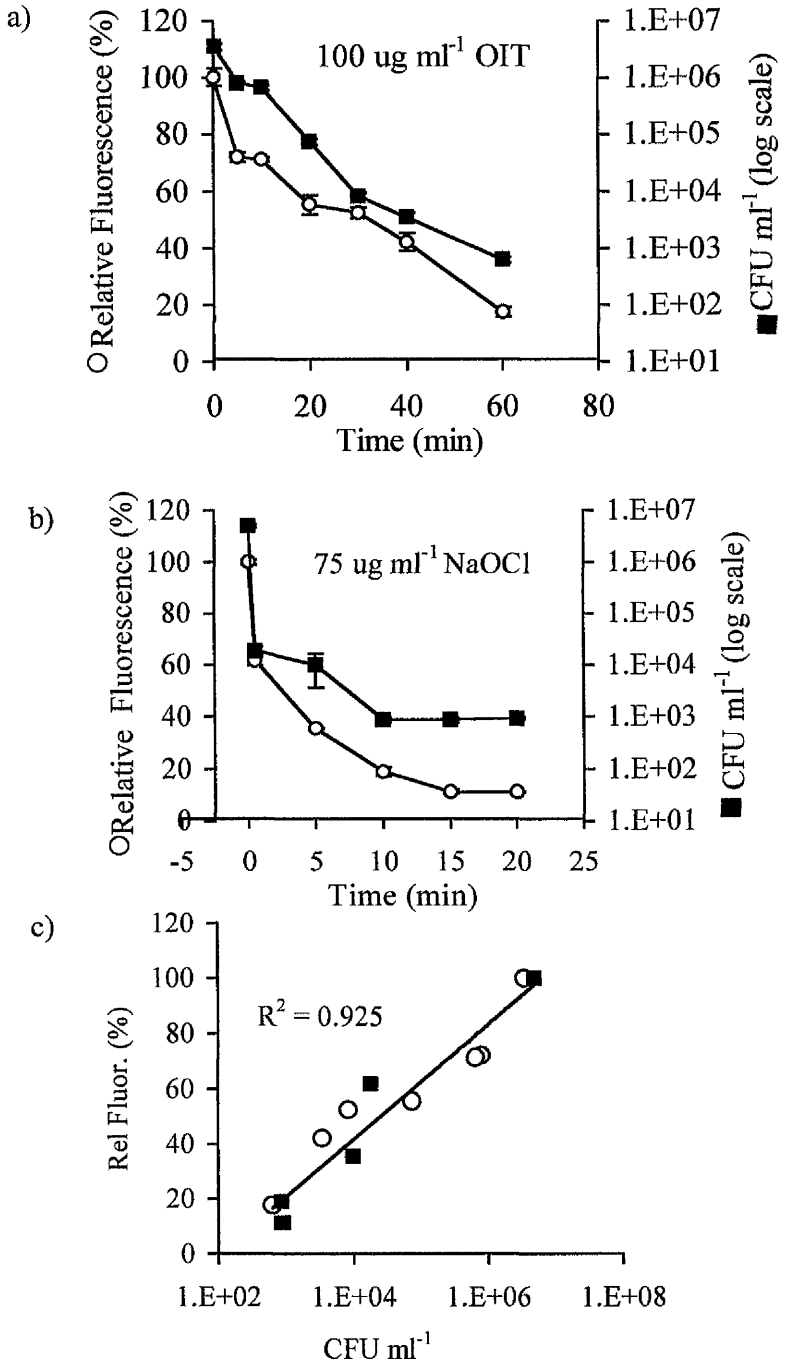

Fig. 3. Influence of the biocides OIT and hypochlorite on loss of GFP fluorescence and cell viability in Ap1 *gfp* blastospores. a) Influence of 100 μg ml^{-1} OIT, b) Influence of 75 μg ml^{-1} hypochlorite; c) Correlation between GFP fluorescence and numbers of viable cells during treatment with OIT (O) and sodium hypochlorite (■).

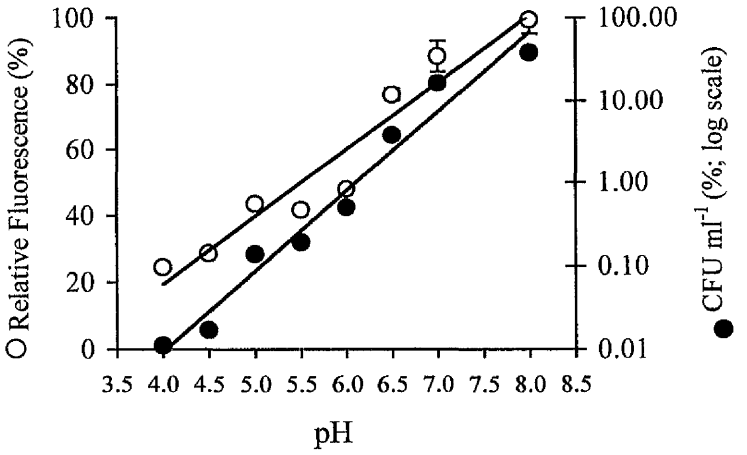

Fig 4. Influence of external pH on the percentage loss of GFP fluorescence and viability of Ap1 *gfp* cells after addition of 100 ppm OIT for 1 h.

Comparison of a range of biocides at in-use concentrations

The kinetics of GFP fluorescence loss in the presence of 5 broad-spectrum biocides commonly incorporated within pPVC were are shown in Fig. 5. All of the biocides caused greater than 60% loss of fluorescence after 4 h, and caused 100% loss of viability within this period. OIT and BBIT caused a complete reduction of fluorescence to baseline levels. Fluorescence levels of cells incubated with DMSO without biocide fell to 90% after 4 h, and 90% (5.4×10^6 CFU ml^{-1}) of cells remained viable after this period.

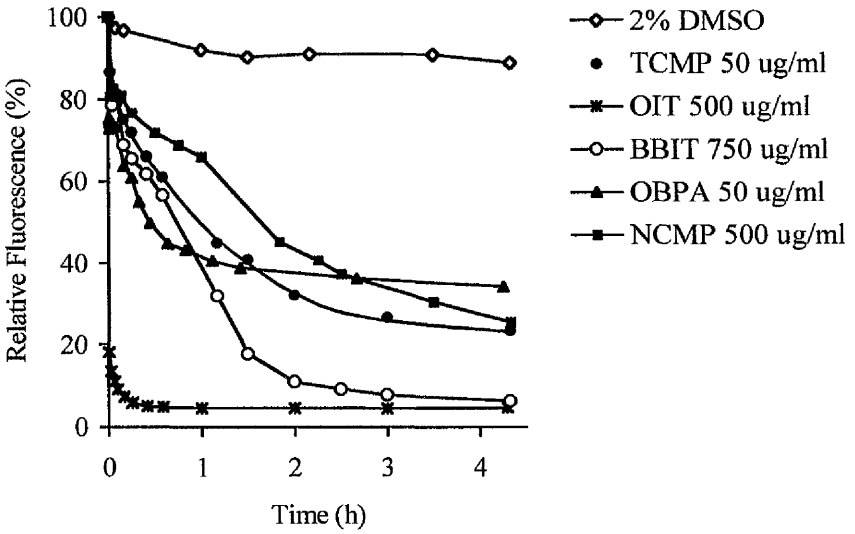

Fig. 5. Influence of a range of biocides on GFP fluorescence in Ap1 *gfp* blastospores. Blastospores were exposed to in-use concentrations of biocide at which they are normally incorporated within pPVC: NCMP 500 μg ml^{-1} (■); OBPA 50 μg ml^{-1} (▲); TCMP 50 μg ml^{-1} (●); BBIT 750 μg ml^{-1} (○); OIT 500 μg ml^{-1} (*); 2% DMSO (no biocide) (◇).

DISCUSSION

This study is the first to demonstrate that GFP fluorescence may be used as a real-time, noninvasive indicator of fungal susceptibility to antimicrobial agents. In bacteria, GFP fluorescence has been shown to be rapidly reduced in the presence of a number of biocides (Casey & Nguyen, 1995; Kremer *et al.*, 1995). Casey & Nguyen (1995) observed a correlation between loss of GFP fluorescence and loss of cell viability in *E. coli*, and suggested that the technique was useful as a rapid screen for antimicrobial compounds. However, no detailed explanation for the loss of GFP fluorescence was provided. Further, because of the inherent stability of GFP, this approach was thought by Collins *et al.*, (Collins *et al.*, 1998) to risk falsely equating GFP fluorescence and viability, potentially leading to some active compounds being overlooked.

Since these studies, GFP has become established as a sensitive and accurate indicator of intracellular pH (Kneen *et al.*, 1998; Llopis *et al.*, 1998; Robey *et al.*, 1998). Intracellular pH is considered to be one of the most important factors in fungal physiology. Intracellular pH regulates the key enzymes in glycolysis and gluconeogenesis (Noshiro *et al.*, 1987; Imai & Ohno, 1995) and is thought to regulate other cell responses, including the induction of heat shock proteins (Weitzel *et al.*, 1987). In *Neurospora crassa*, cytosolic acidification to pH 6.5 using propionic acid resulted in complete inhibition of growth (Parton *et al.*, 1997), and several studies have correlated intracellular pH with fungal cell viability (Imai & Ohno, 1995; Viegas *et al.*, 1998). We propose that the observed correlation between GFP fluorescence and cell viability in this study results from the sensitivity of GFP to intracellular pH.

Intracellular pH is regulated by the essential fungal plasma membrane proton pump H^+ATPase (Serrano *et al.*, 1986; Serrano, 1998). Inhibition of H^+ATPase causes rapid depolarization of the plasma membrane, followed by intracellular acidification and cell death (Ermolayeva & Sanders, 1995; Ben-Josef *et al.*, 2000). Intracellular acidification and GFP fluorescence loss in the presence of biocides may result from one or more of three principal mechanisms: i) direct inhibition of proton extrusion through inactivation of H^+ATPase, ii) indirect loss of H^+ATPase activity through inhibition of respiration or other essential cell processes; iii) non-specific modification of cell permeability. Thus the mechanism of fluorescence loss will depend on the mode of action of the biocide. Isothiozolinone biocides (such as OIT) are membrane active biocides that inhibit cellular sulfhydryl groups (Fuller *et al.*, 1985; Sondossi *et al.*, 1993). H^+ATPase contains cysteine sufhydryl groups which are critical for enzyme function (Monk & Perlin, 1994).

Therefore H^+ATPase in Ap1 *gfp* was probably inhibited by OIT, resulting in direct intracellular acidification and loss of viability. The biphasic loss of GFP fluorescence observed at 100 μg ml^{-1} OIT suggests that this biocide may have more than one mode of action. Sodium hypochlorite is a powerful oxidizing agent that destroys the cellular activity of proteins, and can oxidize sulfhydryl groups to sulphides or sulphoxides (Bloomfield, 1995). Therefore sodium hypochlorite could also inhibit H^+ATPase and cause intracellular acidification. Fungistatic compounds which inhibit growth but do not interfere with intracellular pH regulation would not be expected to cause fluorescence loss in this system. The triazole drug itraconazole caused low levels of fluorescence loss (< 20%) from Ap1 *gfp* cells (data not shown). Since triazole drugs prevent growth by interfering with ergosterol biosynthesis (Baldwin & Wiggins, 1984), it might be expected that intracellular pH and GFP fluorescence are not greatly influenced by this drug.

Other possible causes for loss of GFP fluorescence are leaking of GFP to the external medium due to loss of membrane integrity or cell lysis, or protease degradation of the GFP chromaphore. However, microscopic observation of Ap1 *gfp* cells showed that neither sodium hypochlorite or OIT caused cell lysis. Moreover, loss of GFP fluorescence was not caused by protease activity or leaking of GFP since fluorescence was almost completely reversible when cells were washed and resuspended in pH 7 buffer (data not shown).

A range of biocides normally used in pPVC each caused rapid loss of GFP fluorescence and complete loss of viability from Ap1 *gfp* cells. All of these biocides are thought to be enzyme inhibitors and therefore could cause intracellular acidification through inhibition of H^+ATPase. However, the biocides NCMP, OBPA and TCMP did not cause a complete loss of GFP fluorescence. Each of these biocides showed visible precipitation when added to Ap1 *gfp* cells, and therefore some biocide could remain in contact with the cells throughout centrifugation, washing and plating procedures. This carry-over may result in underestimation of viable cell numbers at the time of sampling, and is a limitation of the plate-count technique. Further studies will determine whether complete loss of viability among Ap1 *gfp* cells can occur without total inhibition of GFP fluorescence. These experiments will also address the possibility of using GFP in MIC-type studies of antifungal susceptibility.

Loss of GFP fluorescence and cell viability were highly dependant on the pH of the external medium. If intracellular pH regulation is inhibited, then it follows that lower external pH values will cause greater intracellular acidification and thus greater loss of fluorescence and viability. However, the activity of many biocides is also dependant on pH. For example, the active entity of sodium hypochlorite is undissociated hypochlorous acid, which is more abundant at low pH values (Trueman, 1971). Therefore we chose to use OIT to investigate the effect of external pH on loss of fluorescence and cell viability as OIT does not possess ionizable groups that would be influenced by pH.

The measurement of GFP fluorescence described in this study proved to be a rapid and simple procedure for monitoring viability in Ap1 *gfp* cells in the presence of biocides. These data suggest that GFP fluorescence in low external pH will prove useful for screening potential fungicidal agents and compounds that inhibit intracellular pH regulation. This technique also has considerable potential as a simple and economic alternative to the use of fluorescent dyes for estimating cell viability. Another obvious advantage of using GFP is that it could indicate biocide susceptibility in real time and with spatial resolution *in-situ* using a fluorescence microscope. Such a system would allow studies of the efficacy of biocides incorporated within substrata without the necessity for the removal of attached microorganisms. Further studies of GFP as a unique indicator of susceptibility to antimicrobial compounds may also provide information about the mechanism of action of existing compounds, thereby allowing a more rational selection of appropriate biocides.

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Chapter 6.

General conclusions and future work

GENERAL CONCLUSIONS AND FUTURE WORK

This study investigated microbial colonization of pPVC in a longitudinal trial *in-situ* and found *Aureobasidium pullulans* to be the principal colonizing organism (Chapter 2). The mechanism of adhesion of *A. pullulans* to pPVC was subsequently characterised (Chapter 3), and novel techniques were developed to study its growth on pPVC (Chapter 4) and biocide susceptibility (Chapter 5) using GFP as a reporter system. The principal conclusions from each of these chapters, as well as the opportunities for future research that have arisen, are summarized below.

Chapter 2. Fungal colonization and biodeterioration of pPVC

A detailed, quantitative investigation of the microbial colonization of pPVC was carried out over a 2 year period. rDNA sequencing was found to be a useful technique for the rapid and unambiguous identification of fungi isolated during the trial. *A. pullulans* was the predominant colonizing organism, establishing on the pPVC after 25 weeks. A number of yeasts and yeast-like fungi, including *Rhodotorula aurantica* and *Kluyveromyces spp.* established much later on the pPVC after 80 weeks of exposure. Bacteria did not colonize the pPVC during the trial.

The colonization sequence was observed to occur independently on three separate exposure racks, and therefore was reproducible during the period of this trial. However, further studies are required to determine whether the same pattern of colonization would occur on different occasions or at different locations. This study was conducted at an industrial location in Manchester. Useful comparisons might therefore be made with pPVC exposed at a rural site. An important aspect of such studies would be the relationship between the composition of the air microflora and the observed colonization sequence at the different sites. The influence of variations in the air microflora both with time, and with location, on the colonization and biodeterioration of pPVC are not known.

The deteriogenic properties of fungi isolated from the pPVC were tested *in-vitro*. All strains of *A. pullulans* tested were able to grow on pPVC as the sole carbon source and could degrade the plasticiser DOA. In contrast, most yeast isolates could not grow on pPVC or degrade DOA. These results support the hypothesis that microbial succession occurs on the pPVC, and that secondary colonizing yeasts require additional nutrients, e.g. the metabolites of *A. pullulans*, or the accumulation of exogenous nutrients, before

they can grow on the pPVC. In general, however, tests for the biodeterioration of pPVC were not predictive of the ability of fungi to colonize pPVC within the environment. For example, strains of *Alternaria alternata*, *Epicoccum nigrum*, and *Cladosporium* sp. were able to grow well on pPVC *in-vitro* but did not establish on pPVC exposed outdoors. Clearly environmental factors play an important role in determining which organisms can colonize pPVC *in-situ*, and these results emphasise the importance of field trials in investigations of the microbial susceptibility of pPVC formulations in their intended applications.

Chapter 3. Plasticisers increase adhesion of *Aureobasidium pullulans* to pPVC

The adhesion assay developed allowed study of the mechanism of adhesion of *A. pullulans* blastospores to uPVC and pPVC. Adhesion to pPVC was greater than that to uPVC by a maximum of 280% after 4-h incubation with 10^8 blastospores ml^{-1}. This enhanced adhesion was confirmed to be due to the plasticisers, since adhesion to uPVC was increased by up to 308% in the presence of a dispersion of DOA and DOP. These plasticisers did not increase adhesion to pPVC by providing a carbon source for *A. pullulans* because killing of cells with sodium azide had no effect on adhesion. Therefore the physico-chemical nature of the enhanced adhesion to pPVC was investigated.

Hydrophobic interactions were found to control adhesion of *A. pullulans* blastospores to uPVC. This was evident from the strong correlation observed between increasing substratum hydrophobicity and increased adhesion to a range of unplasticised polymers including pPVC. In addition, neither the pH nor the electrolyte concentration of the suspension buffer, both of which affect electrostatic interactions, influenced adhesion to uPVC.

In contrast, adhesion to pPVC was controlled principally by electrostatic interactions. Increased adhesion to pPVC occurred despite a reduced susbstratum hydrophobicity in comparison with uPVC. In addition, adhesion to pPVC was strongly dependant on both the pH and electrolyte concentration of the suspension medium, and was maximum at pH 8 and with an electrolyte concentration of 10 mM NaCl. Therefore plasticisers increase adhesion of *A. pullulans* blastospores to pPVC through an interaction mediated by electrostatic forces.

These results suggest that plasticisers may accelerate biodeterioration processes occurring on pPVC not only by providing a carbon source for microorganisms, but also

by enhancing fungal adhesion. Further studies will determine whether plasticisers also increase adhesion of other fungi to the pPVC. It is possible that the relative ability of fungi to adhere to pPVC exerts a major influence on the colonization sequence observed *in-situ*. Thus if enhanced adhesion caused by plasticisers is unique to *A. pullulans*, then this may in part explain the success of this fungus in the colonization of pPVC *in-situ*.

Further studies are required to determine whether the formulation of the pPVC can be modified to reduce attachment of *A. pullulans* or other fungi. For example plasticisers could be chosen that minimize electrostatic interactions in order to reduce adhesion of *A. pullulans*. Alternatively, pPVC formulations could be designed that minimize presentation of plasticisers at the surface of the plastic. Thus, the information provided by this study may be useful in the design of novel pPVC formulations that utilize surface chemistry to reduce microbial attachment.

Chapter 4. Expression of cytosolic GFP in *Aureobasidium pullulans* allows observation and quantification of the fungus on pPVC

In order to develop new techniques to study the colonization of pPVC, *A. pullulans* was transformed to express GFP. All morphotypes of *A. pullulans*, including hyphae, blastospores and chlamydospores expressed cytosolic GFP and fluoresced brightly. Because GFP is intrinsically fluorescent, growth of the transformed strain Ap1 *gfp* was readily visible on the pPVC substratum in real-time under epifluorescence microscopy. Growth of Ap1 *gfp* was also amenable to quantification using image analysis. Therefore GFP expression in *A. pullulans* provides a powerful method to study the interactions of this fungus with the pPVC substratum.

The brightly fluorescent *A. pullulans* strain, in combination with the quantitative techniques offered by image analysis, presents a number of possibilities for future research into the colonization of pPVC. Firstly, factors that influence growth of *A. pullulans* on the pPVC could be investigated with ease *in-vitro* using this technique. For example, the efficacy of biocides, or different plasticiser/additive combinations in preventing *A. pullulans* growth on the pPVC substratum could be compared. The influence of environmental factors, such as temperature and humidity, on *A. pullulans* colonization and growth patterns could also be studied. Secondly, the labelled *A. pullulans* strain may provide an ideal means to study marked populations of the organism on pPVC *in-situ* in nature. For example, tagged strains could be applied to the

pPVC surface to investigate colonization patterns and the interactions of *A. pullulans* with other colonizing microorganisms *in-situ.*

Chapter 5. GFP as a novel indicator of biocide susceptibility in *A. pullulans*

This study demonstrated that GFP fluorescence in *A. pullulans* was useful as a real-time, non-invasive indicator of the susceptibility of the fungus to antimicrobial compounds. The biocides OIT and sodium hypochlorite each caused rapid losses of GFP fluorescence from Ap1 *gfp* cells in a concentration dependant manner at low external pH. Furthermore, loss of GFP fluorescence was highly correlated with a decrease in the number of viable cells ($R^2 > 0.92$). Loss of GFP fluorescence and cell viability was highly dependant on external pH; maximum losses of pH and viability occurred at pH 4 while reduction of GFP fluorescence was absent at pH 8 and was associated with a lower reduction in viability. It is proposed that the observed correlation between GFP fluorescence and cell viability in this study results from the sensitivity of both of these properties to intracellular pH. The technique allowed the relative antimicrobial properties of a range of biocides normally incorporated pPVC to be compared in real-time using simple fluorescence measurements. These data suggest that GFP fluorescence in Ap1 *gfp* cells could be developed for high-throughput screening of potential antifungal agents and compounds.

Further research is required to examine in greater detail the relationship between GFP fluorescence and cell viability in *A. pullulans*. For example, the level of fluorescence at which the cell loses viability is not known. One approach to this question might be to study the proportion of cells within the population that remain fluorescent during biocide treatment. Does the entire population of cells lose fluorescence uniformly, or is there variation between cells in terms of susceptibility to antimicrobial compounds and fluorescence loss? In the latter case it would be possible to conduct germination studies under epifluorescence microscopy in order to compare viability among a mixed population of fluorescent and non-fluorescent cells. This would determine whether a complete loss of fluorescence is necessary to cause loss of viability from the cell.

Future work will also develop GFP fluorescence in *A. pullulans* as a technique to monitor antimicrobial susceptibility on substrata using epifluorescence microscopy and image analysis. Preliminary studies have shown that this technique may be useful in comparing the efficacy of biocides incorporated within pPVC (see Appendix 6, p151), and in comparing the relative antimicrobial susceptibility of surface attached and

planktonic *A. pullulans* cells (see Appendix 7, p154). Thus development of these techniques will allow investigation of whether attached phenotypes of *A. pullulans* are more resistant to biocides than their free-living counterparts.

Appendices

APPENDIX 1. Exposure of pPVC pieces *in-situ*.

In order to study microbial colonization of pPVC *in-situ*, a rack system was constructed to support pPVC samples over the course of a long-term colonization experiment (Chapter 2). Fig. A1a shows the three identical racks constructed on-site at Avecia Biocides, Blackley, North Manchester. Each rack supports up to 300 7 cm × 4.2 cm pPVC pieces (Fig A1b). pPVC pieces were arranged randomly on the rack to enable a 'randomised block' design for statistical comparisons between racks.

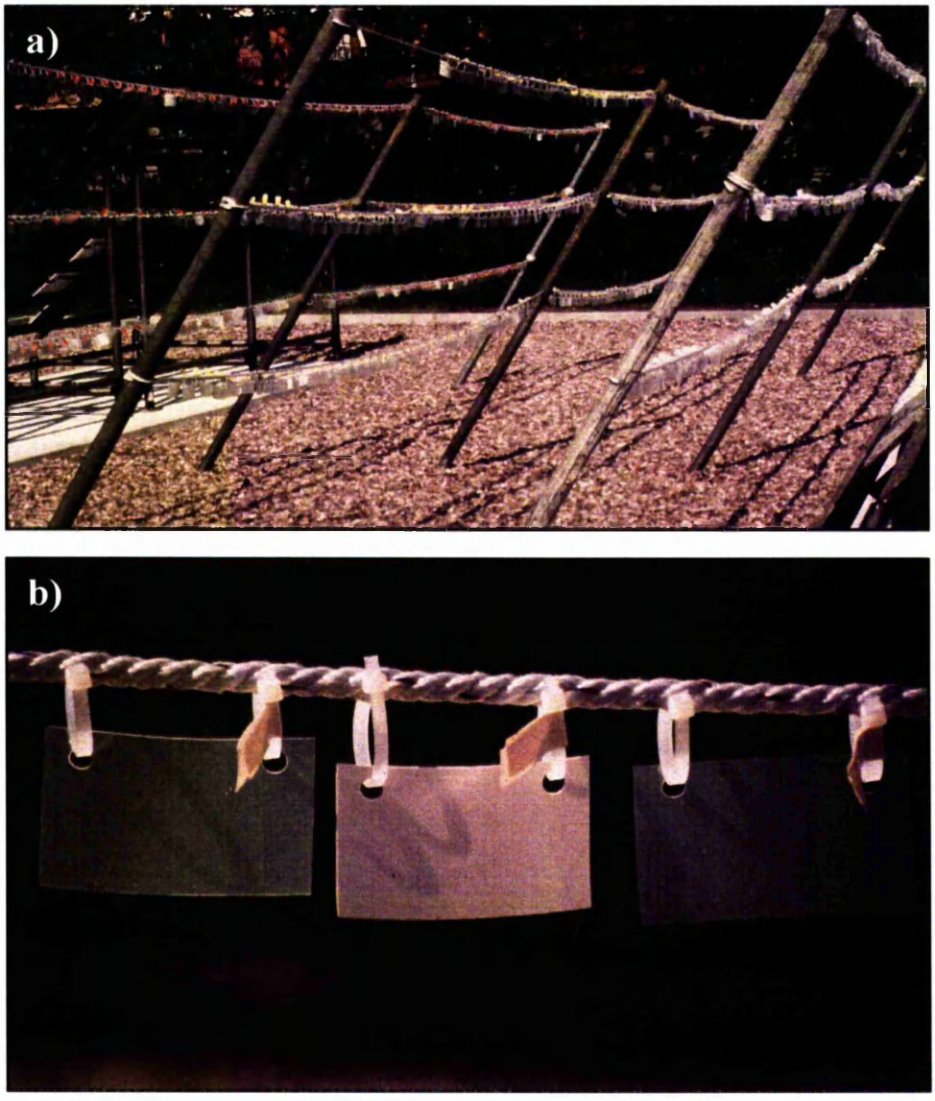

Fig. A1. a) Support racks used for the long-term exposure of pPVC *in-situ*. Each rack consists of 3 × 7 metre lines of 8mm polypropylene wound rope positioned 1.0, 1.5 and 2.0 m above ground level. The three racks are positioned 1 m apart and are orientated at 60° to the horizontal to minimize cross contamination of specimens during rainfall. b) Individual 7 × 4.2 cm pPVC pieces supported by cable ties.

APPENDIX 1 Ctd.

An additional objective of this study was to investigate the efficacy of a range of biocides incorporated into pPVC in preventing microbial colonization. Therefore, In addition to the TiO_2-containing pPVC studied in this project, a large number of pPVC pieces incorporating different biocides were also exposed on the support racks. However, these formulations did not contain TiO_2 pigment which appeared to protect other pPVC pieces from UV and thermal degradation of the plastic. They were thus unstable under environmental conditions and suffered extensive discolouration and plasticiser loss from the pPVC. Consequently the additional samples were not considered in this study.

APPENDIX 2. Measurement of the tensile strength of pPVC

The mechanical properties of pPVC were monitored throughout the *in-situ* trial (Chapter 2). A steel punch was constructed (Mechanical Workshop, Stopford Building, Manchester) to cut dumbbell shaped pieces from samples of pPVC. The punch produced dumbbells with a gauge length of 23 mm and width of 4.1 mm. The thickness of each dumbbell was measured before testing. Tension tests were carried out using a tensiometer (Instrom 4301, Instrom Corp., Massachusetts, USA) fitted with a 100N load cell and with the crosshead speed set to 40 mm min^{-1}. Dumbbell-shapes were gripped at both ends in the machine and stretched, the extension and force required being continually monitored until breaking point (Materials Testing System v4.10c, Instrom Corp.). The tensile strength of pPVC pieces was measured at each sample time throughout the *in-situ* trial. Tensile strength is a measure of the maximum stress a material will sustain before breaking. It is equal to the maximum load divided by the original cross sectional area of the specimen. Prior to exposure to the atmosphere, the mean and standard deviation of tensile strength measurements from 6 separate pPVC pieces was 8.9 $\pm$ 0.7 Mpa. At each sample time, 2 dumbbells were cut from each of three replicate pPVC pieces removed from the sample racks and their tensile strength measured. Statistical analysis of tensile strength measurements using analysis of variance showed that no significant increase in tensile strength of pPVC occurred throughout the *in-situ* trial ($P > 0.05$).

APPENDIX 3. Influence of plasticisers on the water contact angle of uPVC

Plasticisers that leach from the pPVC into the surrounding liquid medium could potentially influence adhesion by acting as surfactants. However, the water contact angle on uPVC, which would be greatly reduced in the presence of a surfactant, was not influenced by the incorporation of a mixed dispersion of DOP and DOA into the wetting fluid. Using a DCA, the contact angle on uPVC measured using deionized water was compared with that obtained using a 50% mixed DOP and DOA plasticiser dispersion. The mean and standard deviation of 5 advancing contact angle measurements using deionized water was 91.8° ± 0.9°. With plasticisers incorporated into the wetting medium, the mean contact angle was 91.3° ± 0.3°. No significant difference in contact angle occurred between these wetting fluids ($P = 0.33$). Therefore the plasticisers DOP and DOA did not influence the water contact angle on uPVC and therefore do not act as surfactants.

APPENDIX 4. Influence of sodium azide on adhesion of *A. pullulans* to pPVC

Plasticisers can be utilized as a carbon source by *A. pullulans*. Consequently DOA and DOP could influence adhesion by stimulating metabolic activity and the synthesis of adhesive cell structures. The influence of sodium azide, a respiratory inhibitor, on adhesion of *A. pullulans* blastospores to pPVC discs was therefore investigated. Blastospores were incubated with 20 mM sodium azide for 6 h. Cells were then washed once in PBS by centrifugation at 3600 × g and the number of viable cells remaining was determined by plating on malt extract agar. No colony forming units (CFUs) were recovered on malt extract agar after azide treatment, indicating 100% loss of viability of *A. pullulans* cells. Adhesion to pPVC was compared relative to untreated blastospores using the adhesion assay. The mean and standard deviation of percentage surface cover of 5 replicate discs with azide treated cells was 39.2% ± 2.6%. Adhesion levels of untreated blastospores to 5 replicate pPVC discs were 36.4% ± 4.0%. Adhesion of killed cells to pPVC were not significantly different to those of control cells ($P = 0.23$), therefore utilisation of plasticisers as a carbon source by *A. pullulans* is unlikely to influence adhesion to pPVC.

APPENDIX 5. Southern hybridization analysis of *A. pullulans* transformants

For Southern hybridizations, *A. pullulans* genomic DNA was prepared according to the method of Anderson *et al.*, (1996) as described in Chapter 2, p62. Wild-type and co-transformant DNA (approx. 2 μg) was digested with *Eco*R1 or *Bam*H1, separated by electrophoresis on 0.6% agarose, and blotted onto a nylon membrane (Roche Molecular Biochemicals, Lewes). A synthetic *gfp* gene with a Ser65Thr substitution and codon optimized for mammalian expression (Fernández-Ábalos *et al.* 1998) was used as the probe. Probe DNA was labelled with digoxigenin (DIG) using the DIG High Prime Kit (Roche). Hybridization and detection of the probe was carried out using the DIG-system according to the manufacturers instructions (Roche). Southern analysis showed that integration of the vector had occurred at a single site in both transformants Ap1 *gfp* and Ap2 *gfp* (Fig. A5).

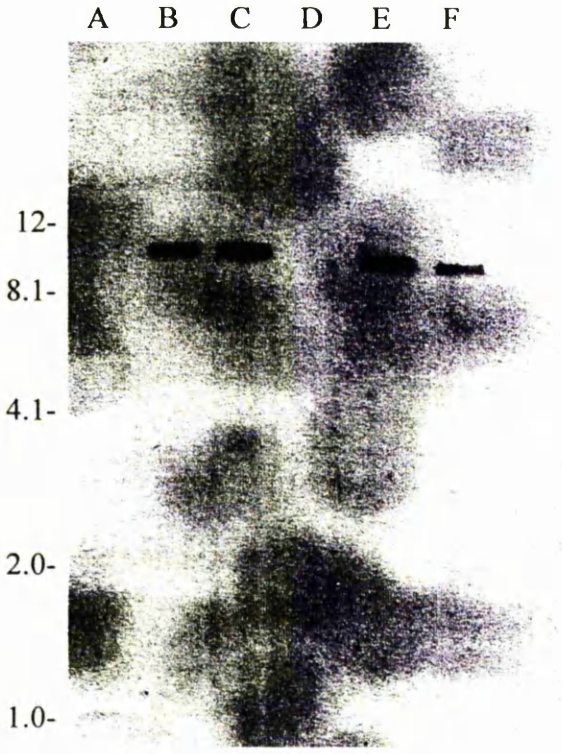

Fig. A5. Southern hybridization analysis of *A. pullulans* wild-type and co-transformed strains. Lanes A, B, and C contain DNA from wild-type, Ap1 *gfp* and Ap2 *gfp* strains, respectively, digested with *Eco*R1. Lanes D, E, and F contain DNA from wild-type, Ap1 *gfp* and Ap2 *gfp* strains, respectively, digested with *Bam*H1. Molecular size markers (Kb) are shown on the left margin.

APPENDIX 5 Ctd. References

Anderson, M. J., K. Gull, and D. W. Denning. 1996. Molecular typing by random amplification of polymorphic DNA and M13 southern hybridization of related paired isolates of *Aspergillus fumigatus*. J. Clin. Microbiol. **34**:87-93.

Fernández-Abalos, J. M., H. Fox, C. Pitt, B. Wells, and J. H. Doonan. 1998. Plant-adapted green fluorescent protein is a vital reporter for gene expression, protein localization and mitosis in the filamentous fungus, *Aspergillus nidulans*. Mol. Microbiol. **27**:121-130.

APPENDIX 6. Influence of biocides incorporated into pPVC on GFP fluorescence in *A. pullulans*

GFP fluorescence in *A. pullulans* is a potential technique to monitor biocide susceptibility on substrata in real-time using epifluorescence microscopy and quantitative image analysis techniques. The influence of a range of biocides incorporated within pPVC on GFP fluorescence in Ap1 *gfp* cells was therefore investigated. pPVC discs (4 mm in diameter) containing biocides at their normal in-use concentrations were placed in to the wells of a 96-well flexible PVC assay plate. Aliquots of Ap1 *gfp* blastospores (200 µl, 1 x 10^4 cells ml^{-1} in citrate-phosphate buffer, pH5) were placed into wells containing the pPVC discs. In order to rapidly and firmly apply cells to the surface of the pPVC, the assay plate was placed into a flat-based centrifuge bucket and centrifuged at 3600 × g for 2 min. Discs were examined under epifluorescence microscopy after 18 h incubation at 25°C (Fig. A6).

Results and conclusions

This study demonstrates that loss of GFP fluorescence caused by biocides can be readily observed on the pPVC substratum using epifluorescence microscopy. When incorporated into pPVC, only NCMP and OIT caused visible losses of GFP fluorescence from Ap1 *gfp* cells (Fig. A6). DCOIT, BBIT and OBPA did not cause fluorescence loss over the 18 h period. In contrast, all of the biocides tested in this study were previously demonstrated to cause rapid (< 4 h) fluorescence losses of greater than 60% when applied as liquids to Ap1 *gfp* cell suspensions (Chapter 5, p132). Thus differences exist between these biocides in the effect of incorporation into pPVC on their ability to cause fluorescence loss from *A. pullulans* cells.

Many factors may influence the efficacy of a biocide when it is incorporated into a substratum. For example, the concentration of biocide presented at the surface of the pPVC may depend upon the chemistry and properties of the formulation, including the migratory ability of the biocide. In addition, only that part of the cell in contact with the substratum may be influenced by the biocide, whereas liquid biocides can act over the

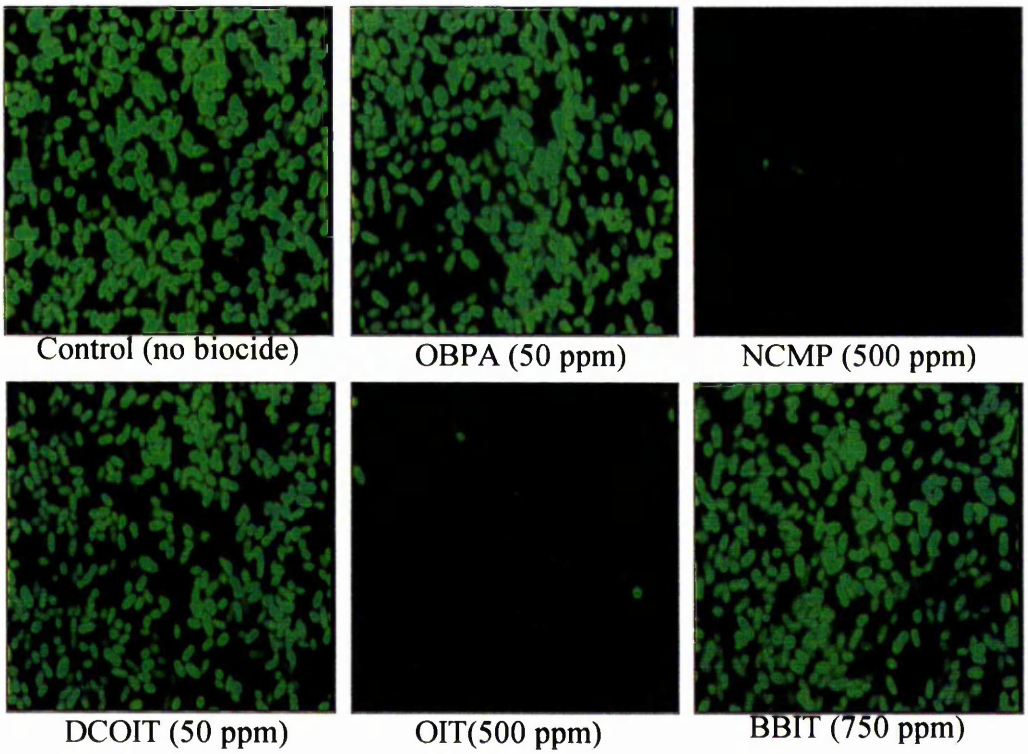

Fig. A6. Effect of biocides incorporated into pPVC on GFP fluorescence in Ap1 *gfp* cells after 18 h incubation under citrate-phosphate buffer (pH 5.0)

whole the cell. A much higher concentration of biocide may therefore be necessary in the substratum to exert the same antimicrobial effect as the biocide in the bulk liquid phase. GFP expression in *A. pullulans* may therefore provide a useful technique to determine the optimum concentration of biocides for incorporation into pPVC.

It is also possible that the organism itself may develop increased antimicrobial resistance in response to surface attachment. Increased antimicrobial resistance within biofilms has been widely studied in bacteria (for a review, see Morton *et al.*, 1998), and has more recently been shown to occur in the yeast *Candida albicans* (Baillie & Douglas, 1998). However, whether this phenomenum is widespread among environmental fungi is not known. Consequently we carried out a preliminary investigation of the influence of surface attachment in *A. pullulans* on GFP fluorescence loss in response to the biocide OIT (see Appendix 7).

Further studies are required to determine whether the observed correlation between GFP fluorescence and cell viability in planktonic cells also occurrs in surface attached Ap1 *gfp* cells. However this study suggests that GFP-labelled cells will provide a powerful method to compare the efficacy of biocides incorporated in pPVC. This will allow a more rational selection of biocides and individual biocide concentrations for the protection of pPVC.

References

Baillie, G. S., and L. J. Douglas. 1998. Iron-limited biofilms of *Candida albicans* and their susceptibility to amphoteracin B. Antimicrobial Agents and Chemotherapy **42**:2146-2149.

Morton, L. H. G., D. L. A. Greenway, C. C. Gaylarde, and S. B. Surman. 1998. Consideration of some implications of the resistance of biofilms to biocides. Int. Biodeter. Biodegrad. **41**:247-259.

APPENDIX 7. Influence of OIT on GFP fluorescence in surface attached and planktonic *A. pullulans* cells.

The influence of the biocide OIT on GFP fluorescence in surface attached and planktonic *A. pullulans* cells was investigated. Ap1 *gfp* biofilms were grown on pPVC for 18 h in malt-extract broth as described (see Chapter 4, p110). pPVC pieces were then washed by dipping twice in deionized water and placed under 20 ml citrate-phosphate buffer (pH 5) in a Petri dish. Planktonic Ap1 *gfp* cells were grown in malt extract broth for 18 h with shaking at 200 rpm, and prepared in citrate-phosphate buffer (pH 5) as described (see Chapter 5, p124). The biocide OIT was added at a concentration of 100 μg ml^{-1} directly to Ap1 *gfp* biofilms or blastospore suspensions in buffer. GFP fluorescence in surface attached and planktonic cells was then observed at various time intervals using epifluorescence microscopy.

Results and conclusions

These preliminary results suggest that surface attached *A. pullulans* cells may be more resistant than planktonic cells to the biocide OIT. Addition of 100 μg ml^{-1} OIT to planktonic Ap1 *gfp* blastospores caused almost complete loss of fluorescence after 40 min incubation (Fig. A7a). In contrast, GFP fluorescence was still readily visible within surface attached cells after 4 h incubation with the same concentration of OIT (Fig. A7b). This represents a 500% increase in the time period over which GFP fluorescence is retained in surface attached cells compared to planktonic cells. Since GFP fluorescence has been correlated with cell viability in *A. pullulans* planktonic cells (see Chapter 5), these data suggest that surface attached *A. pullulans* cells may be more resistant to OIT than their planktonic counterparts.

This difference in susceptibility may be caused by the different culture conditions used for *A. pullulans* biofilm development and batch culture growth (stirred Petri-dish and shake-flask culture respectively). Shake-flask culture may provide higher oxygen levels, and subsequently higher growth rates, which may influence biocide resistance (Evans *et al.*, 1991). Reduced susceptibility to OIT may also result from a lower cell-surface area exposed to the biocide caused by surface attachment. Of most interest, however, is the possibility that distinct phenotypes with increased antimicrobial resistance are expressed in direct response to surface attachment. In *A. pullulans*, a 'switch' from yeast-phase to mycelial-phase growth was always observed on pPVC when blastospores were applied

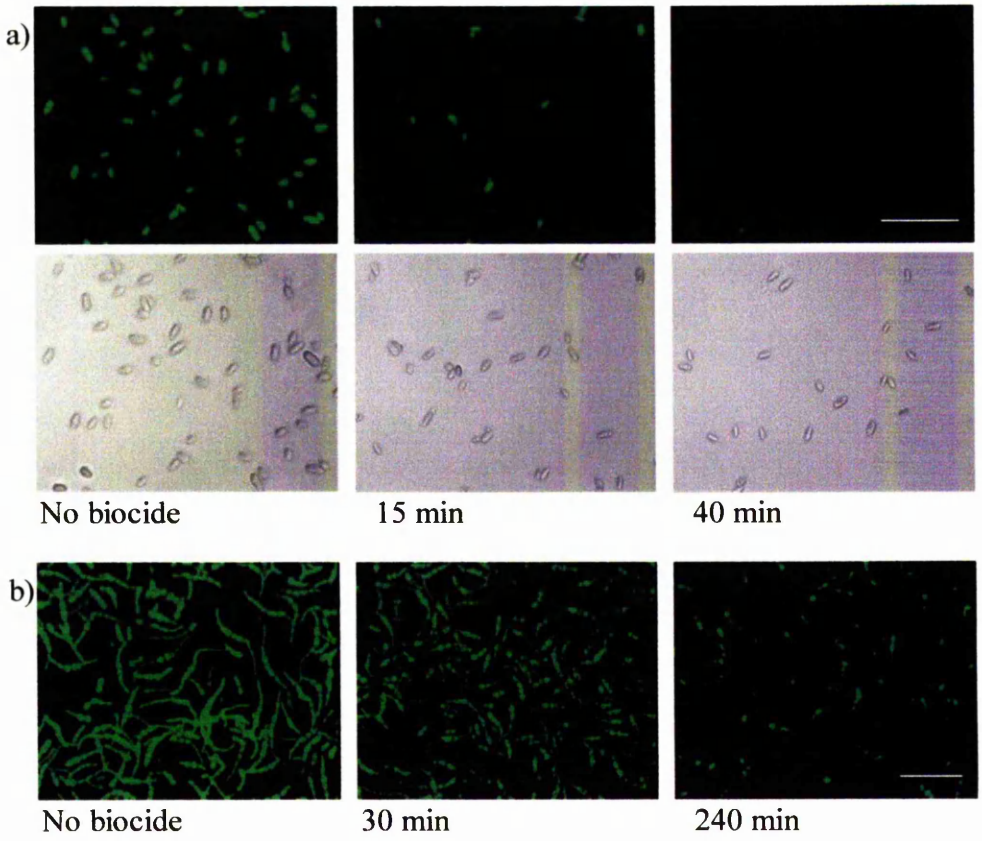

Fig. A7. Influence of OIT (100 μg ml^{-1}) on GFP fluorescence in surface attached and planktonic Ap1 *gfp* cells in buffer (pH 5). Bar = 20 μm. a) GFP fluorescence (upper), and corresponding light field images (lower), of planktonic blastospores over time. b) GFP fluorescence in Ap1 *gfp* cells attached to pPVC.

to the substratum (see Chapter 4). Physiological or metabolic differences between yeast-like and hyphal cells may influence the susceptibility of *A. pullulans* to pPVC. Increased biocide resistance in surface attached cells of a fungus has recently been demonstrated to occur in *Candida albicans* (Baillie & Douglas, 1998). This study suggests that the phenomenum may also occur in *A. pullulans* during the colonization of pPVC.

Further studies are required to determine whether GFP fluorescence and cell viability are correlated for surface attached cells as well as for planktonic cells. However, this study suggests that GFP-labelled cells will provide a powerful technique to study the influence of surface attachment on antimicrobial susceptibility in *A. pullulans*.

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Evans, D. J., M. R. W. Brown, D. G. Allison, and P. Gilbert. 1990. Susceptibility of bacterial biofilms to tobramycin: role of specific growth rate and phase in the division cycle. J. Antimicrobial Chemotherapy **25**:585-591.

Morton, L. H. G., D. L. A. Greenway, C. C. Gaylarde, and S. B. Surman. 1998. Consideration of some implications of the resistance of biofilms to biocides. Int. Biodet. Biodegd. **41**:247-259.

APPENDIX 8. Published papers and abstracts

Papers in international refereed journals

J. S. Webb, M. Greenhalgh, H. C. Van der Mei, S. J. Read, I. M. Eastwood, M. Nixon, G. D. Robson & P. S. Handley. 1999. Plasticisers increase adhesion of the deteriogenic fungus *Aureobasidium pullulans* to pPVC. *Applied and Environmental Microbiology,* 65: 3575-81.

J. S. Webb, M. Nixon, I. M. Eastwood, M. Greenhalgh, G. D. Robson, and P. S. Handley. 2000. Fungal colonization and biodeterioration of plasticised polyvinyl chloride. *Applied and Environmental Microbiology*. In press.

J. S. Webb, S. R. Barratt, M. Nixon, I. M. Eastwood, M. Greenhalgh, G. D. Robson, and P. S. Handley. Submitted. GFP as a novel indicator of antimicrobial susceptibility in *Aureobasidium pullulans. Applied and Environmental Microbiology.*

Other Publications

J. S. Webb, G. D. Robson, M. Nixon, I. M. Eastwood, M. Greenhalgh and P. S. Handley. 1999. Colonization of plasticised PVC by *Aureobasidium pullulans.* p105-111. *In* 'Biofilms: The Good, the Bad and the Ugly' Proceedings of the Fourth Meeting of the Biofilm Club, Gregynog Hall, Powys, Wales. 18-20th September 1999.

J. S. Webb. 1999. Growth of *Aureobasidium pullulans* on plasticised PVC using GFP as a reporter. 'Images of Biology' series, cover photograph. *Biological Sciences Review* 12(8).

Abstracts

J. S. Webb., M. Nixon, I. M. Eastwood, M. Greenhalgh, G. D. Robson and P. S. Handley. 1999. Adhesion and biofilm formation of the deteriogenic fungus *Aureobasidium pullulans* occurring on PVC. *99th General Meeting of the American Society for Microbiology, Chicago, Illinois, May 1999.*

J. S. Webb., M. Nixon, I. M. Eastwood, M. Greenhalgh, G. D. Robson and P. S. Handley. 1998. Fungal colonization and adhesion processes occurring on plasticised polyvinylchloride. *Biofilm Club one-day workshop meeting: 'Biofilms in the environment', Manchester, November 1998.*

J. S. Webb., M. Nixon, I. M. Eastwood, M. Greenhalgh, G. D. Robson and P. S. Handley. 1998. Microbial colonization of plasticised polyvinylchloride. *Eighth International Symposium for Microbial Ecology, Halifax, Nova Scotia, August 1998.*

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Plasticizers Increase Adhesion of the Deteriogenic Fungus *Aureobasidium pullulans* to Polyvinyl Chloride

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Initial adhesion of fungi to plasticized polyvinyl chloride (pPVC) may determine subsequent colonization and biodeterioration processes. The deteriogenic fungus *Aureobasidium pullulans* was used to investigate the physicochemical nature of adhesion to both unplasticized PVC (uPVC) and pPVC containing the plasticizers dioctyl phthalate (DOP) and dioctyl adipate (DOA). A quantitative adhesion assay using image analysis identified fundamental differences in the mechanism of adhesion of *A. pullulans* blastospores to these substrata. Adhesion to pPVC was greater than that to uPVC by a maximum of 280% after a 4-h incubation with 10^8 blastospores ml^{-1}. That plasticizers enhance adhesion to PVC was confirmed by incorporating a dispersion of both DOA and DOP into the blastospore suspension. Adhesion to uPVC was increased by up to 308% in the presence of the dispersed plasticizers. Hydrophobic interactions were found to dominate adhesion to uPVC because (i) a strong positive correlation was observed between substratum hydrophobicity (measured by using a dynamic contact angle analyzer) and adhesion to a range of unplasticized polymers including uPVC, and (ii) neither the pH nor the electrolyte concentration of the suspension buffer, both of which influence electrostatic interactions, affected adhesion to uPVC. In contrast, adhesion to pPVC is principally controlled by electrostatic interactions. Enhanced adhesion to pPVC occurred despite a relative reduction of 13° in the water contact angle of pPVC compared to that of uPVC. Furthermore, adhesion to pPVC was strongly dependent on both the pH and electrolyte concentration of the suspension medium, reaching maximum levels at pH 8 and with an electrolyte concentration of 10 mM NaCl. Plasticization with DOP and DOA therefore increases adhesion of *A. pullulans* blastospores to pPVC through an interaction mediated by electrostatic forces.

Major problems of substratum damage occur when plasticized polyvinyl chloride (pPVC) is colonized by microorganisms in many different environmental situations. It has long been established that this susceptibility results from the presence of plasticizers, commonly organic acid esters such as dioctyl phthalate (DOP) and dioctyl adipate (DOA), added to modify physical or mechanical properties of the polymer (7). Since these early studies, degradation of ester-based plasticizers has been demonstrated among both bacteria (4, 5, 14) and fungi (2, 45). Loss of plasticizers from pPVC due to microbial degradation results in brittleness, shrinkage, and ultimately failure of the PVC in its intended application.

Although no detailed quantitative studies have been published, fungi are reported to be the principal deteriogenic organisms in structural and outdoor applications of pPVC (3, 18). However, despite widespread commercial use of pPVC and considerable economic losses due to its biodeterioration, mechanisms of fungal attachment to pPVC have not previously been examined.

Microbial adhesion is the first in a series of events that occur during the colonization of a solid substratum. Adhesion to inert materials such as plastics or glass is known to be controlled by nonspecific interactions between the cell surface and the substratum. Research has focused on bacterial adhesion, where hydrophobicity both of the substratum (8, 35, 46) and of the cell surface (33, 40, 44) and electrostatic charge on the cell surface (17, 34, 43) are important factors in adhesion to inert substrata.

While bacterial adhesion to surfaces has been studied extensively, the nonspecific adhesion of fungi has received comparatively little attention. Most fungal adhesion studies have focused on adhesion of the opportunistic pathogen *Candida albicans* to synthetic materials used for medical prostheses. Increased adhesion of *C. albicans* to plastics has been associated with increased hydrophobicity both of the fungal surface (30, 32) and of the substratum (27). Electrostatic forces have also been demonstrated to influence adhesion of *C. albicans* to hydrophilic glass (25), although they are thought to be of minor importance in adhesion to more hydrophobic plastics (27). Therefore, similar physicochemical characteristics appear to control adhesion of both bacteria and *C. albicans*.

Nothing is known about the physicochemical factors controlling adhesion of fungi that colonize and deteriorate plastics within the environment. This study reports on the mechanisms of adhesion of the deuteromycete *A. pullulans* because it was found to be the dominant fungus causing deterioration of pPVC films during outdoor exposure trials in Florida (18). *A. pullulans* is ubiquitous within the environment and is known to colonize many habitats (9). It is one of relatively few fungi that can colonize living leaf surfaces (1) and is also the principal colonizer of painted wood surfaces (15).

As part of a long-term study of microbial colonization processes occurring on pPVC, the initial adhesion of the deterio-

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genic fungus *A. pullulans* has been studied in vitro. We have investigated the effect of incorporating the plasticizers DOP and DOA into PVC on the adhesion of *A. pullulans* blastospores. Physicochemical parameters influencing the nonspecific adhesion of blastospores to both unplasticized PVC (uPVC) and pPVC have also been investigated.

MATERIALS AND METHODS

A. pullulans (de Bary) Arnaud. *A. pullulans* (IMI70103) was maintained on malt extract agar (Oxoid, Unipath, Ltd., Basingstoke, United Kingdom), and to produce blastospores, cultures were incubated for 5 days at 25°C in the dark. Under these conditions, only the mycelial and blastospore morphotypes of *A. pullulans* developed. For long-term storage, blastospores were frozen at −80°C in 20% (vol/vol) glycerol solution (BDH, Poole, United Kingdom).

Growth of *A. pullulans* on pPVC as the sole carbon source was used as a test method to confirm the ability of this isolate to deteriorate pPVC. A piece of pPVC (4 by 4 cm) was sandwiched between two layers of 12 g of bacteriological agar liter^{-1} (Oxoid) in deionized water. The upper layer of agar was seeded with approximately 10^5 blastospores of *A. pullulans* ml^{-1} before pouring. A control agar plate was prepared that contained blastospores, but without a piece of pPVC incorporated. Both plates were incubated at 25°C for 7 days, after which time, growth of *A. pullulans* could clearly be seen in agar immediately above the pPVC. Absence of growth on the control plate confirmed the ability of *A. pullulans* to obtain a source of carbon from the pPVC.

Adhesion assay. pPVC sheets (0.5 mm thick) were formulated that contained the following (parts per hundred resin): EP 6779 (PVC resin) [European Vinyls Corporation (UK) Ltd.], 75; Vinnolit C65V (PVC resin), 25; DOP (plasticizer) (Exxon Chemicals, Ltd.), 25; DOA (plasticizer) (Exxon Chemicals, Ltd.), 25; Lankromark LN138 (calcium-zinc stabilizer) (AKCROS), 2; and Lankroflex ED63 (epoxidized oleate ester), 3. uPVC sheets (0.2 mm thick) were obtained from Goodfellow, Ltd., Cambridge, United Kingdom.

Discs, 4 mm in diameter, were cut from sheets by using a pair of punch pliers (RS components). Discs were cleaned in 2% Lipsol detergent (LIP, Shipley, England) and rinsed thoroughly in deionized water. Handled by the edges only and with forceps, discs were placed in rows of five into the wells of a 96-well assay plate (Costar).

A. pullulans blastospores were harvested in phosphate-buffered saline (PBS) containing 8.0 g of NaCl liter^{-1}, 0.2 g of KCl liter^{-1}, 1.15 g of Na_2HPO_4 liter^{-1}, 0.2 g of KH_2PO_4 liter^{-1}, and 0.1 g of $MgCl_2$ liter^{-1} at pH 7.3 and separated from hyphae by filtration through three layers of lens tissue paper. Blastospores were centrifuged for 8 min at 3,600 × *g*, washed three times, and resuspended in PBS to an optical density at 540 nm of 0.59 (10^8 blastospores ml^{-1}). Aliquots of 200 μl of the blastospore suspension were placed into wells containing pPVC discs by using a multichannel pipette (Treff Lab). Following incubation at 25°C, the blastospore suspension was removed by pipette, and discs were washed three times by sequentially adding and removing 200-μl aliquots of PBS. During the washing procedure, pipette tips were inserted into the base of wells so that liquid crossed the surfaces of discs evenly and with minimal variation in shear forces. Spores were then fixed for 15 min by the addition of 100 μl of 20% (vol/vol) formaldehyde to each well and washed once in PBS. Discs were allowed to air dry for 1 h before being stained with Gram crystal violet (Difco). Stain (100 μl) was applied to each well for 5 s and quickly removed, and discs were washed a further three times in PBS. Stained discs were then transferred from wells to a microscope slide for image analysis.

Blastospores on pPVC discs were visualized by using a Leica Medilux microscope equipped with an automated stage for image analysis. Digital images of the pPVC surface were captured under bright-field illumination with a charge-coupled device camera (Sony XC-75CE), and the percentage of surface covered with attached blastospores was quantified with image analysis software (Quantimet Qwin 570, version 01.00; Leica, Ltd. Cambridge, United Kingdom).

Reproducibility of adhesion data. To determine disc-to-disc (intrabatch) variation within the adhesion assay, rows of five discs of both uPVC and pPVC were exposed to a suspension of 10^8 blastospores ml^{-1} derived from a single plate. Five rows were sampled for each material, and adhesion was quantified after a 4-h incubation period. To determine whether different batches of blastospores gave different levels of adhesion (interbatch variation), adhesion was measured by exposing rows of five pPVC and uPVC discs to blastospores harvested from five different cultures. Inter- and intrabatch variation in adhesion were statistically assessed by analysis of variance.

Kinetics of adhesion to uPVC and pPVC. To determine the time period required for maximal adhesion to pPVC, a time course experiment was carried out over a 10-h period with a suspension of 10^8 blastospores ml^{-1}. After each sample time, five pPVC discs were removed, washed, and stained, and the mean percentage of surface cover with blastospores for the discs was determined. The influence of blastospore concentration on numbers of blastospores attaching was determined by exposing rows of five discs to blastospore concentrations in the range 2×10^7 to 5×10^8 blastospores ml^{-1} for a 4-h adhesion period.

Influence of plasticizers on adhesion to uPVC. A mixed dispersion of both DOP and DOA was created within PBS suspension buffer to examine the influence of plasticizers on adhesion of *A. pullulans* blastospores to uPVC. Five milliliters of each plasticizer was added to 400 ml of PBS. Both DOP and DOA are immiscible with water but are completely miscible with each other and form an organic phase over the PBS. The entire volume was homogenized (Ystral D-7801; 260 W; 25,000 rpm; Dottingen) for 1 min and centrifuged for 10 min at 3,600 × *g* in order to remove large droplets of plasticizer. The resulting dispersion was separated from liquid DOP and DOA remaining on the surface of the PBS by running the volume through a glass separation funnel fitted with a tap. The eluent from the column was considered to be the 100% plasticizer concentration. Dilutions were prepared to contain relative plasticizer concentrations in the range 0 to 100% of this undiluted dispersion. Blastospores were suspended to 10^8 blastospores ml^{-1} in each dilution of the dispersion and applied to rows of five discs of uPVC for 4 h. Plasticizer concentrations in PBS were kept constant throughout the washing procedure.

Both DOP and DOA exhibit low water solubility of up to 1 mg liter of H_2O^{-1} (Chemical Abstract Service no. 117-84-0 for DOP and 103-23-1 for DOA). To determine the effect of low levels of dissolved plasticizer on adhesion, dispersed plasticizers in PBS were ultracentrifuged at 80,000 × *g* for 30 min to remove undissolved plasticizer. The resulting clear solution was separated from the remaining liquid DOP and DOA by using a separation funnel, and the eluent from the column was considered to be the 100% dissolved plasticizer concentration. Dilutions were prepared to contain relative dissolved plasticizer concentrations in the range 0 to 100% of the undiluted solution. Rows of five discs of uPVC were exposed for 4 h to blastospores suspended to 10^8 blastospores ml^{-1} in each dilution. Dissolved plasticizer concentrations in PBS were kept constant throughout the washing procedure.

Effect of substratum hydrophobicity on adhesion. Contact angle measurements were made on a range of polymers and glass to examine the influence of substratum hydrophobicity on adhesion. The polymers used were polyethylene tetraphthalate (PET), polypropylene, polytetrafluoroethylene, and uPVC (Goodfellow Ltd.); polyethylene and fluoroethylenepropylene (Fluorplast, Raamsdonkveer, The Netherlands); and tissue culture-treated PET (Thermanox) (Agar Scientific, Ltd., Stansted, United Kingdom). Glass microscope slides were obtained from Chance Proper, Ltd., Warley, England.

Surface hydrophobicity of materials was determined with a DCA-312 dynamic contact angle analyzer (Cahn Instruments, Madison, Wis.) (11). This equipment uses the principle of the Wilhelmy balance and has an advantage over sessile drop methods, in that larger surface areas may be sampled (28). Samples (2 by 2 cm) were cut from sheets of each polymer, and the contact angle was measured by immersion to a depth of 1 cm at a stage speed of 19.6 μm s^{-1}. The wetting fluid used was deionized water (Elix 3; Millipore Corp., Watford, United Kingdom). Advancing contact angles (θ_a) were determined on five replicate samples of each material.

Six replicate discs of each polymer were prepared as described for pPVC and uPVC. Fragments of glass small enough to be inserted into plate wells were prepared by breaking microscope slides under tissue paper. Adhesion to the different materials was measured at a blastospore concentration of 10^8 cells ml^{-1} incubated with discs for 4 h.

Influence of pH on adhesion to pPVC and uPVC. Blastospores were suspended to 10^8 blastospores ml^{-1} in PBS adjusted to pH values in the range 2 to 13. Rows of five discs of both pPVC and uPVC were exposed for 4 h to blastospores at each pH value. pH values in the PBS solutions were kept constant throughout the washing procedure.

Influence of electrolyte concentration on adhesion to pPVC and uPVC. Blastospores were suspended to 10^8 blastospores ml^{-1} in deionized water containing the electrolyte NaCl in the concentration range 0 to 100 mM. uPVC and pPVC discs in rows of five were exposed to blastospores at each NaCl concentration for 4 h. NaCl electrolyte concentrations were kept constant throughout the washing procedure.

Microelectrophoresis. Zeta potentials, a measure of the net charge on the surface of the blastospores, were measured in PBS at a range of pH values at room temperature with a Lazer Zee Meter 501 (PenKem), which uses the scattering of incident laser light to detect cells at relatively low magnifications. The absolute electrophoretic mobilities can be derived directly from the velocities of the organisms in the applied electric field, the applied voltage, and the dimensions of the electrophoresis chamber (21). Electrophoretic mobilities were measured for blastospores suspended to a concentration of ≈1 × 10^7 blastospores ml^{-1} and converted into zeta potentials on the basis of the Helmholtz-Smoluchowski equation (20).

LTSEM. For low-temperature scanning electron microscopy (LTSEM), pPVC and uPVC discs were incubated with 10^8 blastospores ml^{-1} for 4 h before undergoing washing, fixation, and crystal violet staining as under normal assay conditions. The discs were then rapidly frozen by being plunged into nitrogen slush and transferred to a Cambridge 200 scanning electron microscope.

Once inside the microscope, ice on the surface of the discs was sublimed at −65°C until all visible ice crystals had disappeared. The discs were withdrawn into the prechamber and sputter coated with gold. The specimen stub was returned to the cold stage set at −170°C and observed.

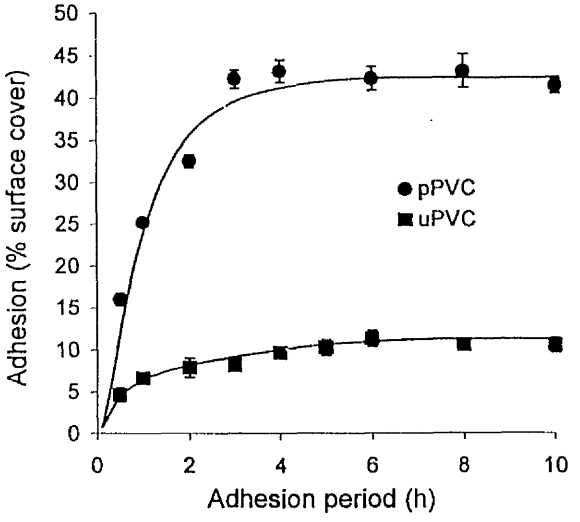

FIG. 1. Time course of adhesion of *A. pullulans* IMI70103 blastospores to uPVC and pPVC. Error bars show ±1 standard error of the mean.

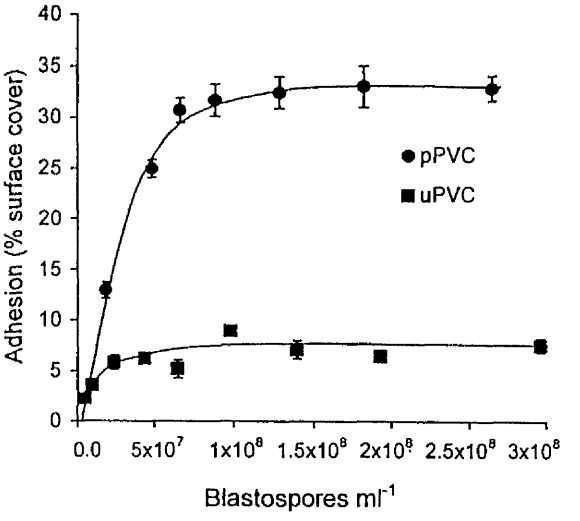

FIG. 2. Influence of blastospore concentration on adhesion of *A. pullulans* to uPVC and pPVC. Error bars show ±1 standard error of the mean.

RESULTS

Adhesion assay. To determine the reproducibility of the adhesion assay, adhesion values were compared statistically by analysis of variance. Disc-to-disc (intrabatch) variation was quantified for both uPVC and pPVC by comparison of adhesion values derived from discs incubated with the same batch of *A. pullulans* blastospores. No significant disc-to-disc variation ($P > 0.05$) occurred for either material among the means of five rows of five discs incubated in the wells of a tissue culture plate. The individual mean and standard deviation values for percentage surface cover with blastospores among the five rows ranged between 32.3% ± 2.3% and 39.5% ± 2.7% for pPVC and between 2.5% ± 0.4% and 3.4% ± 1.4% for uPVC.

However, for both uPVC and pPVC, significant interbatch variation ($P < 0.001$) occurred among mean adhesion values from five separate batches of blastospores. Individual mean and standard deviation values for percentage surface cover with blastospores among each of the five batches ranged between 31.5% ± 3.1% and 43.2% ± 3.0% for pPVC and between 2.4% ± 0.4% and 15.9% ± 3.0% for uPVC. To eliminate this source of variation, each subsequent adhesion experiment was completed with blastospores from a single batch.

Kinetics of adhesion to pPVC and uPVC. The kinetics of adhesion of *A. pullulans* blastospores to pPVC and uPVC were examined by monitoring the percentage of surface cover of discs with adhered blastospores over a 10-h period (Fig. 1). Maximum adhesion to pPVC was 280% greater than that to uPVC at 4 h. Adhesion to uPVC rose quickly to 7% in 1 h and then more slowly to reach a plateau of 11.3% by 6 h. In contrast, the percentage of the pPVC disc surface covered with attached blastospores increased rapidly up to 3 h and reached a maximum of 43% surface cover after 4 h of incubation. Regular checks for blastospore germination on both uPVC and pPVC surfaces were made throughout the 10-h incubation period, and no incidence of germination was observed. A second time course experiment investigated the possibility that the adhesion plateau observed on pPVC was an artifact of the settling process which occurred during incubation and that more blastospores could potentially attach. After a 6-h adhesion period, unbound spores were washed from discs and replaced with a fresh suspension of 10^8 blastospores ml^{-1} in PBS. Adhesion was then monitored for an additional 6-h period. Replacement of the spore suspension resulted in no further increase in adhesion (data not shown), and the percentage of surface cover did not increase beyond a maximum of 43%, suggesting that saturation of binding sites on the pPVC had occurred. Since maximal adhesion on pPVC occurred after 4 h, this time was subsequently chosen as the incubation period.

In order to determine the effect of increasing spore concentration on adhesion, the percentage of cover of pPVC and uPVC discs was determined after 4 h at spore concentrations in the range 5.2×10^6 to 3×10^8 blastospores ml^{-1} (Fig. 2). The isotherm for binding of *A. pullulans* to pPVC showed that as the concentration of unbound spores increased to 7×10^7 blastospores ml^{-1}, adhesion to pPVC increased quickly to 30.6% surface cover, slowed over the concentration range 7×10^7 to 1.3×10^8 blastospores ml^{-1}, and reached a maximum of 33.0% surface cover at a concentration of 1.8×10^8 blastospores ml^{-1}. The percentage of cover of the pPVC surface with blastospores did not exceed 33% (1.1×10^5 blastospores mm^{-2}) in this experiment. Adhesion to uPVC increased rapidly to 5.8% surface cover in the concentration range 5.2×10^6 to 2.3×10^7 blastospores ml^{-1} and reached a plateau of 7.6% surface cover with blastospore concentrations of 1.0×10^8 ml^{-1} and above.

Influence of plasticizers on adhesion. To determine whether the increased adhesion to pPVC relative to uPVC was due to an attractive interaction between blastospores and the plasticizers, adhesion to uPVC was measured with blastospores suspended in a range of concentrations of a mixed dispersion of DOP and DOA (Fig. 3). Adhesion of *A. pullulans* blastospores to uPVC was increased by up to 308% by incorporation of the plasticizers into the suspension medium. Adhesion increased most rapidly within the plasticizer concentration range 0 to 15% of the undiluted dispersion, where the percentage of surface cover of the uPVC with blastospores increased from

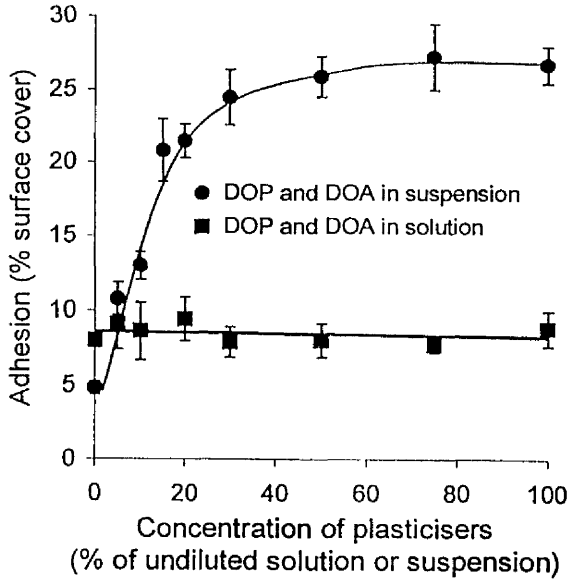

FIG. 3. Influence of the plasticizers DOP and DOA both as a suspension and dissolved in PBS on adhesion of *A. pullulans* blastospores to uPVC. Error bars show ±1 standard error of the mean.

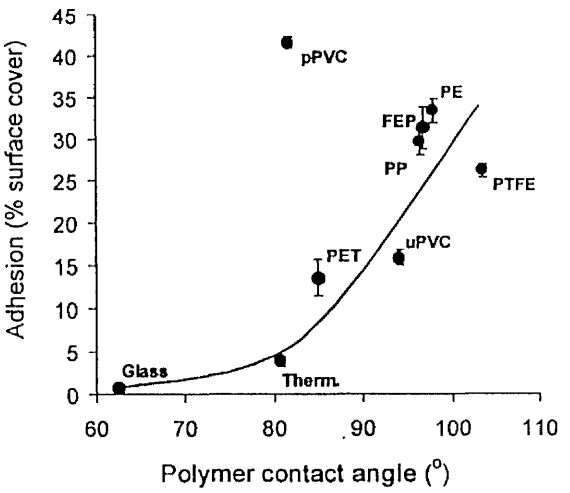

FIG. 4. Effect of increasing surface contact angle on adhesion of *A. pullulans* IMI70103 to different substrata. Therm., Thermanox (tissue culture-treated PET); PTFE, polytetrafluoroethylene; PP, polypropylene; FEP, fluoroethylene polypropylene; PE, polyethylene. Error bars show ±1 standard error of the mean.

6.2% to 20.7%. The rate of increase in adhesion slowed during the plasticizer concentration range of 15 to 50%, reaching a plateau in adhesion of 25.4% surface cover with blastospores when the relative concentration of plasticizers was 50% of the undiluted dispersion. While adhesion to uPVC was strongly dependent on the concentration of dispersed plasticizers present in PBS, dissolved plasticizers remaining after removal of the dispersion did not influence the attachment of blastospores to the plastic (Fig. 3). Adhesion remained constant at 8% surface cover across the relative dissolved plasticizer concentration range of 0 to 100% of the undiluted solution.

Substratum hydrophobicity. The importance of substratum hydrophobicity in blastospore adhesion was investigated (Fig. 4) by comparing levels of adhesion to a range of materials with different water contact angles. The lowest adhesion was to the relatively hydrophilic glass, where the percentage of cover of the surface with blastospores did not exceed 1%. The highest adhesion levels of 41% surface cover were observed on pPVC. A clear relationship existed between increasing water contact angle of the surface and increased adhesion of blastospores for the majority of materials tested, except pPVC. Adhesion to pPVC was approximately 660% higher than would be expected due to its hydrophobicity. In contrast, adhesion of blastospores to uPVC fitted the relationship between adhesion and hydrophobicity. Despite a relative reduction of 13° in the water contact angle of pPVC compared to that of uPVC, adhesion to pPVC was 163% greater than to uPVC.

Influence of pH and electrolyte concentration. To investigate whether incorporation of plasticizers into PVC influences electrostatic interactions between blastospores and the substratum, levels of adhesion to both pPVC and uPVC were compared at a range of different pHs and electrolyte concentrations of the suspension buffer. Adhesion to pPVC was strongly influenced by pH (Fig. 5), with maximum adhesion occurring in the pH range 6 to 10. The percentage of surface cover with blastospores rose from 12% to 35% as pH increased from 2 to 8, reducing to 14% surface cover at pH 13. In contrast, adhesion of blastospores to uPVC was unaffected by changing the pH of the suspension buffer.

Increasing molarities of NaCl were added to suspensions of blastospores in deionized water prior to incubation with pPVC discs (Fig. 6). The percentage of cover of disc surfaces with attached blastospores increased from 6% with no electrolyte to a maximum of 30% across the optimal concentration range of 6 to 12 mM NaCl. A subsequent reduction in adhesion to 13% surface cover occurred as the electrolyte concentration was further increased to 100 mM. Adhesion to uPVC was unaffected by the electrolyte concentration within the suspension buffer.

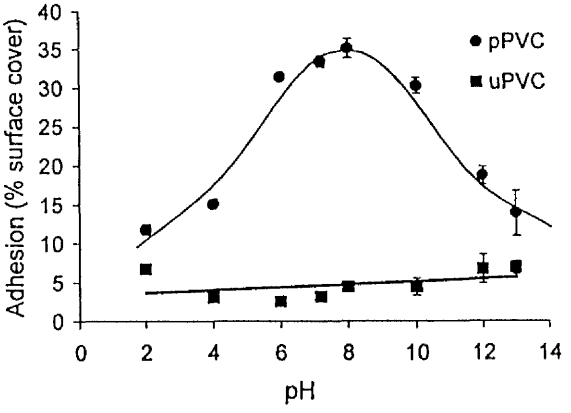

FIG. 5. Influence of suspension buffer pH on adhesion of *A. pullulans* blastospores to pPVC and uPVC. Error bars show ±1 standard error of the mean.

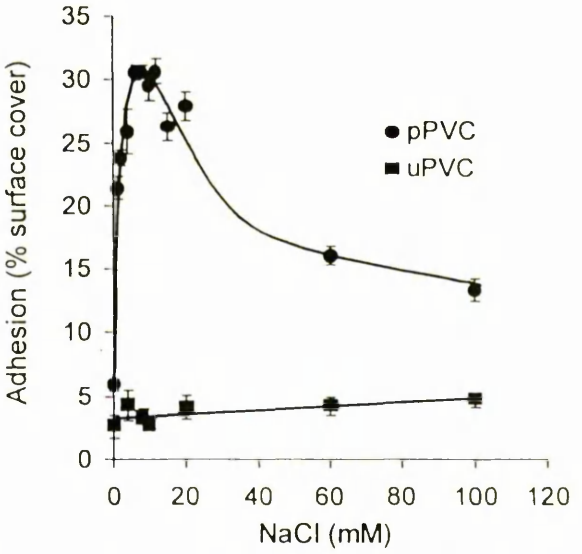

FIG. 6. Effect of electrolyte (NaCl) concentration on adhesion of *A. pullulans* IMI70103 to pPVC and uPVC. Error bars show ±1 standard error of the mean.

Influence of pH on blastospore cell surface charge. To study the effect of pH the on cell surface electrostatic properties of *A. pullulans* blastospores, the zeta potentials of the blastospores were measured in PBS as a function of pH (Fig. 7). The blastospores demonstrated pH-dependent zeta potentials and possessed an isoelectric point within the pH range used, approximately at pH 5. Zeta potentials ranged from +13 mV (pH 2) to −13 mV (pH 12).

LTSEM of pPVC and uPVC with attached blastospores. Blastospores were attached to uPVC and pPVC discs by incubation with 10^8 blastospores ml^{-1} for 4 h. Discs were examined under LTSEM subsequent to the normal washing, fixation, and staining procedures within the adhesion assay (Fig. 8). Blastospores were observed to be randomly dispersed on the surface of the discs and occurred as either single cells or aggregated into clumps. However, there was a far greater density of blastospores on the surface of pPVC (Fig. 8a) than on that of uPVC (Fig. 8b). The surfaces of both the uPVC and pPVC discs appeared smooth under LTSEM.

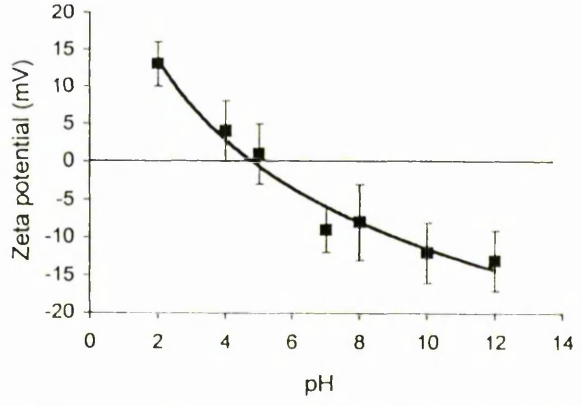

FIG. 7. Zeta potentials of *A. pullulans* IMI70103 blastospores in PBS as a function of pH. Data points represent averages for duplicate zeta potential measurements. Error bars show ±1 standard deviation of the mean.

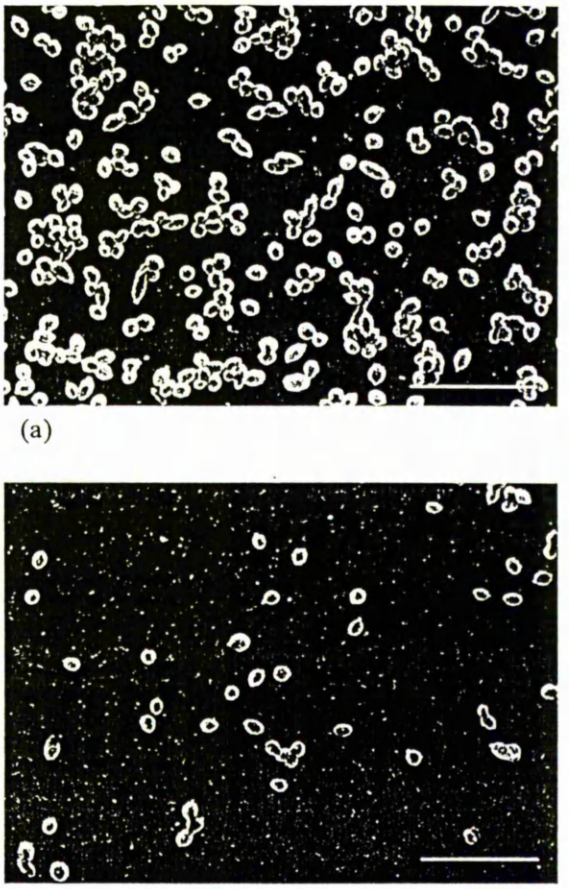

FIG. 8. LTSEM of blastospores attached to pPVC (a) and uPVC (b) discs following incubation with 10^8 blastospores ml^{-1} for 4 h. Discs were washed, fixed, and stained as normal for the adhesion assay. Bar, 20 μm.

DISCUSSION

The plasticizers DOP and DOA clearly enhance adhesion of *A. pullulans* blastospores to PVC, and the physicochemical basis for this enhanced adhesion has been elucidated by using a rapid and quantitative adhesion assay. The plasticizers increased adhesion of blastospores to uPVC by a maximum of 308% when presented as a colloidal suspension (Fig. 3), indicating that there is an affinity of the blastospores for DOA and DOP. Equally high levels of adhesion occurred when blastospores were exposed to uPVC discs pretreated with plasticizer suspension for 1 h (data not shown). Thus, the plasticizers are probably coating the uPVC surface, resulting in an increase in adhesion of blastospores mediated by DOP and DOA in a concentration-dependent manner. On the basis of these observations, subsequent experiments were designed to investigate

the nature of the interaction of blastospores with both uPVC and pPVC.

Plasticizers can be utilized as a carbon source by *A. pullulans*. Consequently, DOA and DOP could increase adhesion indirectly by stimulating metabolic activity and the synthesis of adhesive cell surface structures. The ability of some fungi to attach to substrata is affected by their exposure to respiration inhibitors (26, 38). However, 20 mM sodium azide, a mitochondrial respiration inhibitor, had no apparent effect on the adhesion of *A. pullulans* blastospores to pPVC but caused a 100% reduction in viability of the blastospores (data not shown). Therefore, use of plasticizers as a carbon source by *A. pullulans* is unlikely to contribute to the increased levels of adhesion observed in the presence of plasticizers.

Plasticizers could also influence adhesion indirectly by leaching from the pPVC and dissolving in the liquid phase. Leached plasticizers could alter the physicochemical properties of the blastospore cell surface or of the suspension medium, for example, by acting as surfactants. The ability of plasticizers to leach from pPVC into an aqueous environment is well established (31, 47). However, dissolved plasticizers did not influence adhesion of *A. pullulans* to uPVC (Fig. 3). Furthermore, the water contact angle on uPVC, which would be greatly reduced in the presence of a surfactant, was not influenced by the incorporation of a mixed dispersion of DOP and DOA into the wetting fluid (data not shown). Therefore, the plasticizers do not act as surfactants, and the quantities of plasticizer that may leach from the surface of the pPVC are insufficient to cause the observed difference in adhesion to pPVC and uPVC. Thus, it is likely that plasticizers enhance adhesion by directly influencing physicochemical interactions, such as hydrophobic or electrostatic forces, between blastospores and the PVC substratum.

Hydrophobic interactions control adhesion of *A. pullulans* blastospores to the unplasticized polymers studied, including uPVC. This is evident from the strong positive correlation between increasing substratum hydrophobicity and adhesion observed among the range of polymers tested. Furthermore, electrostatic interactions do not play a detectable role in the adhesion of *A. pullulans* blastospores to uPVC, because adhesion was not influenced by either the pH or the electrolyte concentration of the suspension medium. The importance of substratum hydrophobicity in fungal adhesion to polymers is now well recognized. Increased substratum hydrophobicity has also been shown to correlate with increased adhesion of ungerminated conidia of plant pathogenic fungi (12, 38, 41) and of yeast cells of *C. albicans* (19, 27) to various substrata. However, attachment of blastospores to pPVC clearly did not fit the relationship between substratum hydrophobicity and adhesion, suggesting that electrostatic interactions may play an additional role in blastospore adhesion to pPVC.

Evidence for the involvement of electrostatic forces in adhesion to pPVC comes from the effect of pH on adhesion. pH influences the cell surface charge of blastospores (Fig. 7) and also exerts a major effect on their adhesion to pPVC, which was optimal at pH 8 and minimal at pHs 2 and 13. pPVC containing the plasticizers DOP and DOA has been shown to have a net negative surface charge at pH 7.4 in PBS (23). Therefore, reduced adhesion at pH values above 8 is presumably due to electrostatic repulsion between negatively charged blastospores and a negatively charged pPVC substratum. Support for this hypothesis comes from the observation that adhesion increased concurrently with a reduction in the negative zeta potential of the blastospores in the pH range 6 to 12. However, high levels of adhesion were also predicted at pH values in the range 2 to 5 due to electrostatic attraction between positively charged blastospores and the pPVC surface. The observed inhibition of adhesion at low pH values suggests that other factors controlling adhesion, such as the electrostatic charge of the substratum, are also influenced by the changes in pH of the suspension medium. Reduced adhesion at both high and low pH values has previously been described in the adhesion of bacteria to stainless steel (39, 42) and was interpreted to be caused by changes in surface charge of both the bacterial cell and the substratum. For example, it is possible that surface charge may be altered by hydrolysis of the plasticizers to their free acids at extremes of pH. Furthermore, measurements of the zeta potential at the surface of capillary tubes have demonstrated that several polymers, including PVC, may acquire a net positive charge at low pH values (37). In our study, it is possible that both the spores and the pPVC are protonated at low pH and that adhesion is inhibited due to electrostatic repulsion between the two positively charged surfaces.

Further evidence in support of electrostatic interactions between blastospores and pPVC is provided by the effect of the electrolyte concentration on adhesion. A major reduction in adhesion was observed when the blastospores were suspended in deionized water. This reduction was presumed to be due to electrostatic repulsion, which is more pronounced in solutions of low ionic strength. Similar inhibition of adhesion at low ionic strength has previously been demonstrated among both bacteria (35, 39) and *C. albicans* (25, 27). Maximum adhesion to pPVC occurred at 0.01 M NaCl, at which point electrostatic repulsion between the blastospore and the pPVC was presumed to be at a minimum. The subsequent decrease in adhesion at NaCl concentrations above 0.01 M may result from reduced electrostatic interaction caused by high concentrations of electrolyte. Depending on the characteristics of the adhesive interaction, electrolytes may inhibit adhesion by screening short-range electrostatic attraction between oppositely charged groups (16) or by modifying the conformation of cell surface molecules involved in adhesion (36). Either process could be responsible for the observed reduction in adhesion of blastospores to pPVC, although further study would be required to understand in detail the exact nature of the electrostatic interaction.

The adhesion assay identified significant batch-to-batch variability in adhesion of *A. pullulans* blastospores to PVC. While a number of assays to quantify adhesion of fungal conidia to polymer surfaces have previously been described (6, 12, 29), none have fully investigated the reproducibility of adhesion to the substrata tested. Interbatch variation has previously been determined in studies of bacterial adhesion to polymer surfaces (22, 24), but this study is the first to highlight the fact that similar variation can occur among levels of adhesion of fungal conidia to different substrata. The reasons for variation in adhesion levels between batches are poorly understood, but could be due to slight fluctuations in environmental conditions during growth and development of the blastospores. In practice, since adhesion levels of *A. pullulans* blastospores were consistent within batches, valid comparisons of adhesion data could be made within each batch.

The kinetics of adhesion of *A. pullulans* to uPVC and pPVC suggest that both of these surfaces contain a finite number of binding sites which become saturated at blastospore concentrations above 10^8 blastospores ml^{-1}. SEM observations of blastospores on pPVC and uPVC indicate that these sites are distributed evenly over the substratum. Adhesion data are frequently interpreted in terms of the number of sites which are available for a microorganism to attach to a surface (10, 13). However, saturation of the surface with blastospores may also imply negative cooperativity, i.e., that the presence of attached

blastospores reduces the probability of others attaching in their vicinity. Negative-negative charge interactions that would occur between pPVC and blastospores and between blastospores and blastospores would be expected to create a condition of negative cooperative binding. Negative cooperativity of this nature has previously been shown to occur in the adhesion of *C. albicans* to PET coverslips (27).

In summary, we have demonstrated that incorporation of plasticizers into PVC enhances adhesion of blastospores of the deteriogenic fungus *A. pullulans* through an interaction that is mediated by electrostatic forces. In contrast, adhesion to uPVC is controlled principally by hydrophobic attraction. The implication of these results is that plasticizers may accelerate the biodeterioration processes occurring on pPVC by enhancing fungal adhesion. Such information should be taken into account in the design of novel PVC formulations that utilize surface chemistry to reduce microbial attachment.

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Fungal Colonization and Biodeterioration of Plasticized Polyvinyl Chloride

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Significant substratum damage can occur when plasticized PVC (pPVC) is colonized by microorganisms. We investigated microbial colonization of pPVC in an in situ, longitudinal study. Pieces of pPVC containing the plasticizers dioctyl phthalate and dioctyl adipate (DOA) were exposed to the atmosphere for up to 2 years. Fungal and bacterial populations were quantified, and colonizing fungi were identified by rRNA gene sequencing and morphological characteristics. *Aureobasidium pullulans* was the principal colonizing fungus, establishing itself on the pPVC between 25 and 40 weeks of exposure. A group of yeasts and yeast-like fungi, including *Rhodotorula aurantiaca* and *Kluyveromyces* spp., established themselves on the pPVC much later (after 80 weeks of exposure). Numerically, these organisms dominated *A. pullulans* after 95 weeks, with a mean viable count ± standard error of 1,000 ± 200 yeast CFU cm^{-2}, compared to 390 ± 50 *A. pullulans* CFU cm^{-2}. No bacterial colonization was observed. We also used in vitro tests to characterize the deteriogenic properties of fungi isolated from the pPVC. All strains of *A. pullulans* tested could grow with the intact pPVC formulation as the sole source of carbon, degrade the plasticizer DOA, produce extracellular esterase, and cause weight loss of the substratum during growth in vitro. In contrast, several yeast isolates could not grow on pPVC or degrade DOA. These results suggest that microbial succession may occur during the colonization of pPVC and that *A. pullulans* is critical to the establishment of a microbial community on pPVC.

Plasticized PVC (pPVC) is highly susceptible to microbial attack in many different environmental situations. The problem was first identified in U.S. government reports of the deterioration of military equipment (8, 44), and subsequent reports described defacement and deterioration of commercial pPVC products (20, 50). Biodeterioration of pPVC is now known to occur in a wide range of industrial, commercial, and structural applications (18, 19, 22).

The susceptibility of pPVC results from the presence of plasticizers, commonly organic acid esters such as dioctyl phthalate (DOP) and dioctyl adipate (DOA), added to modify physical or mechanical properties of the polymer. Both bacteria (6, 7, 14) and fungi (5, 36) can degrade ester-based plasticizers. Loss of plasticizers from pPVC due to microbial degradation results in brittleness, shrinkage, and ultimately failure of the pPVC in its intended application.

Microbial deterioration of pPVC has been studied extensively in vitro. Many studies have examined the resistance of pPVC formulations incorporating biocides to colonization by test organisms (38, 39, 46). Other research has determined biodegradability by measuring changes in the physical properties of pPVC, such as changes in tensile strength (49), mass (9), or electrical properties (42) during biodegradation. Several international standard test methods for microbiological susceptibility of plastics have been established (1, 2, 26).

Colonization processes occurring on pPVC in the environment have received comparatively little attention. Nothing is known about the temporal sequence of microbial colonization of pPVC in situ. Existing studies have examined fungal defacement of pPVC in tropical or subtropical climates (24, 40). In both studies fungal growth was evaluated with a subjective, visual assessment of defacement of the pPVC. Neither study examined the role of bacteria in the colonization process. Further, unrecognized fungal growth was normally identified only to genus level with basic morphological techniques. Recently, RNA gene (rDNA) sequencing has been used as a rapid and reliable tool for the identification of fungi to the species level (23). This technique has not been used to identify microorganisms that colonize and deteriorate pPVC in the environment.

We examined the microbial colonization of pPVC in situ by exposing pPVC to the atmosphere in a longitudinal experiment. The principal objectives of this work were (i) to investigate colonization processes, (ii) to identify important deteriogenic organisms to the species level using rDNA sequencing, and (iii) to determine if there was a relationship between the microbial colonization sequence observed in situ and the ability of microorganisms to cause biodeterioration of pPVC in laboratory tests.

MATERIALS AND METHODS

Culture media and maintenance. Fungi and yeasts enriched from pPVC exposed to the atmosphere were maintained on malt extract agar (MEA) (Oxoid, Basingstoke, United Kingdom), a medium used widely for the detection, isolation, and enumeration of fungi. Bacteria were maintained on R2A medium (35) (Difco, Detroit, Mich.), which contains low concentrations of organic nutrients and is used routinely for the enrichment of bacteria from oligotrophic environments. For long term storage at −80°C, fungal spores were harvested from agar plates and frozen in 20% (vol/vol) glycerol. The basal mineral salts medium (MSM) used for determining the deteriogenic properties of organisms contained the following (in grams · liter of distilled H_2O^{-1}): K_2HPO_4, 7; KH_2PO_4, 3; $MgSO_4 \cdot 7H_2O$, 0.1; and $(NH_4)_2SO_4$, 1. DOA agar, used for the isolation of organisms able to degrade the plasticizer DOA, contained MSM supplemented with 2 ml of DOA $liter^{-1}$ and 15 g of bacteriological agar (Oxoid) $liter^{-1}$. For preparation of DOA agar, medium including the DOA was autoclaved at 121°C for 15 min and allowed to cool to approximately 50°C. An emulsion of plasticizer was then created within the medium using a homogenizer at full power for 2 min (260 W, 25,000 rpm; model D-7801; Ystral, Hemel Hempstead, United King-

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dom). Plates were poured immediately after homogenization. DOA liquid medium was prepared as DOA agar except without agar.

Plasticized PVC. Sheets of pPVC, 0.5 mm thick, were formulated that contained the following components (parts per hundred resin): EP 6779 PVC resin, (European Vinyls Corporation Ltd., Runcorne, United Kingdom), 75; Vinnolit C65V PVC resin (Vinnolit, Cologne, Germany), 25; DOP plasticizer (Exxon Chemicals, Southampton, United Kingdom), 25; DOA plasticizer (Exxon Chemicals), 25; Lankromark LN138 calcium-zinc stabilizer, (Akcros Chemicals, Burnley, United Kingdom), 2; Lankroflex ED63 epoxidized oleate ester (Akcros Chemicals), 3; and titanium dioxide pigment (Tioxide Europe, Grimsby, United Kingdom), 10. Individual pPVC pieces were 4.2 by 7 cm and had two 6-mm-diameter holes in the corners of one of the long edges for attachment to in situ support racks.

Exposure of pPVC in situ. Three replicate support racks were constructed on-site at Avecia Biocides (Blackley, Manchester, United Kingdom). Each rack consisted of three lines of 8-mm-diameter polypropylene wound rope (Stevecraft, Manchester, United Kingdom), positioned 1.0, 1.5, and 2.0 m above ground level and held between two steel poles set into concrete and at 7 m apart. Nylon cable ties (4-mm width; RS Components, Corby, United Kingdom) were inserted through the rope windings to support pPVC pieces at numbered locations on the rack. Each rack supports up to 300 pPVC pieces which are held free-hanging in order to eliminate fixed-orientation effects. The three racks were positioned in parallel 1 m apart and were orientated at 60° to the horizontal to limit cross contamination of specimens during rainfall. Ten pieces of pPVC were positioned on each of the three racks at locations chosen using a random number table.

At each sampling time, three replicate pPVC pieces were selected at random, one from each rack. Each piece was cut into three 4.2-by-2.5-cm sections, and a separate analysis was carried out on each section.

Viable counts of fungi, bacteria, and DOA-degrading microorganisms. pPVC sections (4.2 by 2.5 cm) were placed into 25-ml universal tubes (32-mm diameter; 80-mm length) containing 10 ml of sterile distilled H_2O. The tubes were shaken vigorously in an automatic side-arm shaker (Gallenkamp, Leicester, United Kingdom) for 1 min. Plasticized PVC samples were transferred to a petri dish containing 5 ml of H_2O and scraped heavily three times on both sides using the flat edge of a sterile scalpel blade. The pPVC, H_2O, and scalpel blade were then returned to the universal tubes and shaken for a further minute using the sidearm shaker. A dilution series to 10^{-3} was prepared from each universal tube. Aliquots of 0.2 ml from each dilution were spread onto three replicate plates each of malt extract, R2A, and DOA agar. Viable counts were performed on MEA plates after 5 days of incubation at 25°C, on R2A plates after 7 days of incubation, and on plasticizer agar plates after 14 days of incubation. To investigate whether statistically significant changes in CFU counts occurred between sample times, overall mean viable counts for three replicate pPVC pieces at each time point were compared using analysis of variance.

Viable counts of fungi within the atmosphere. As a control, to determine whether DOA-degrading organisms could be isolated from the atmosphere, plates of both MEA and DOA agar media were exposed to the atmosphere at a height of 20 m on the roof of the Stopford Building, Manchester, United Kingdom, in March 1997 (during United Kingdom's winter). Three replicate plates of each medium were retrieved after 20 h of exposure to the atmosphere. The number of fungal CFU on each medium was counted after 3, 5, and 7 days of incubation at 25°C.

Scanning electron microscopy (SEM). pPVC pieces exposed in situ for 95 weeks were rapidly frozen in liquid nitrogen. Specimens were freeze-dried overnight (model BSA; BOC Edwards, Crawley, United Kingdom). Specimens were attached to stubs using Electrodag 915 (Acheson Industries, Reading, United Kingdom) and sputter coated (model S150 device; BOC Edwards) with gold before being examined using a Stereoscan 360 scanning electron microscope (Cambridge Instruments, Cambridge, United Kingdom).

Identification of fungi isolated from pPVC. Fungal isolates were identified by PCR amplification and partial sequencing of the internally transcribed spacer (ITS) regions and the 5.8S rDNA or of the V3 domain of large subunit (28S) rDNA. Initially only the ITS region was sequenced. However, when this sequence was insufficient to establish identity, the V3 region also was sequenced.

For preparation of genomic DNA, fresh mycelia or *Saccharomyces cerevisiae* cells were harvested in deionized water from overnight plate cultures grown on MEA. The biomass was pelleted by centrifugation at 8,000 × *g* for 10 min, and the supernatant was discarded. The pellet was frozen by placing the centrifuge tube into liquid nitrogen, and tubes were then stored at −80°C until the pellet was ground in a mortar under liquid N_2. DNA was extracted according to the method of Anderson et al. (3). DNA was observed following electrophoresis in 1% agarose in TPE buffer (90 mM Tris-phosphate, 2 mM EDTA) and staining with ethidium bromide (1 μg ml^{-1} in TPE buffer).

The V3 variable region at the 5′ end of the 28S rDNA was amplified with the fungal universal primers V3-1 (5′ GCATATCAATAAGCGGAGGAAAAG) and V3-2 (5′ GGTCCGTGTTTCAAGACGG) (16). PCR reagent concentrations were 0.2 μM for primers V3-1 and V3-2, 2.5 mM $MgCl_2$, 200 μM for each of the four deoxynucleoside triphosphates, and 1.25 U of *Taq* DNA polymerase (Roche Diagnostics Ltd., Lewes, United Kingdom) per 50-μl reaction mixture. Amplification was performed for 30 cycles with denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. ITS regions were amplified using fungal universal primers ITS-1 (5′ TCCGTAGGTGAACCTGCGG) and ITS-4 (5′ TCCTCCGCTTATTGATATGC) (45). PCR reagent concentrations were as for V3-1 and V3-2 with the exception of concentrations of 0.25 μM for primers ITS-1 and ITS-4 and 1.5 mM for $MgCl_2$. Amplification was performed for 35 cycles with denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min. Amplified products were purified using the QIAquick PCR purification kit (Qiagen Ltd., Crawley, United Kingdom).

Both strands of the amplified products were sequenced using the ABI BigDye Dideoxy Terminator Cycle Sequencing kit (Applied Biosystems Inc., Warrington, United Kingdom). Cycle-sequencing conditions were denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 4 min for 25 cycles, with a final extension at 60°C for 4 min. The annealing temperature was increased to 55°C for sequencing reactions using the V3-1 primer. Forward and reverse sequences were aligned using ABI Autoassembler software (Applied Biosystems Inc.), and the overlapping consensus sequence was compared with sequences in the EMBL fungal DNA database using Fasta3 sequence homology searches. Isolates that were identified using their rDNA sequences were compared with published descriptions of colony and conidial morphology using microscopic examination. A number of isolates, including those for which no rDNA identity was found, were identified using morphological techniques at the International Mycological Institute at CABI Bioscience, Egham, United Kingdom.

In vitro tests for biodeterioration of pPVC. We characterized the deteriogenic properties of fungi isolated from pPVC and two additional strains of *Aureobasidium pullulans*. *A. pullulans* IMI70103 was obtained from CABI Bioscience, Egham, United Kingdom, and *A. pullulans* PRAFS8 was provided by Avecia Biocides, Manchester, United Kingdom.

(i) **Preparation of inocula.** Spores or yeast cells were harvested from MEA plates in deionized water and filtered through three layers of lens tissue paper (Whatman, Maidstone, United Kingdom). Suspensions were washed three times in deionized water by centrifugation at 12,000 × *g* for 8 min and then adjusted to approximately 10^6 spores or yeast cells ml^{-1} in deionized water using a hemocytometer. Suspensions were checked for purity by streaking onto MEA.

(ii) **Biomass and extracellular esterase production with DOA as sole carbon source.** Isolates were grown in DOA liquid medium for 2 weeks at 25°C and shaken at 250 rpm. Each 100-ml flask, containing 40 ml of medium, was inoculated with 0.2 ml of spore or yeast suspension. Three replicate flasks were inoculated with each microorganism. For esterase assays, 1.5-ml samples of culture fluid were removed using a syringe, clarified by filtration through a 0.2-μm-pore-size cellulose-nitrate filter (Sartorius, Epsom, United Kingdom), and then either stored at −20°C or used immediately for assays. Cultures of filamentous fungi were then filtered through preweighed filter paper (Whatman no. 1) and dried at 70°C until a constant weight was reached. Yeast cultures were decanted to preweighed 50-ml centrifuge tubes and centrifuged at 3,600 × *g* for 5 min to pellet cells. The supernatant was discarded, and tubes were incubated at 70°C until a constant weight was reached, usually 24 to 36 hours.

Nonspecific esterase activity was determined by a spectrophotometric assay with *p*-nitrophenol butyrate (PNB) (Sigma) as substrate (17). Hydrolysis of PNB yields *p*-nitrophenol, which absorbs maximally at 400 nm under alkaline conditions. The assay mixture (1 ml volume) contained 2.2 mM PNB in sodium acetate buffer (50 mM; pH 5.5) in cuvettes. Culture fluid (500 μl) was added, and cuvettes were incubated at 25°C for 1 h. The reaction mix was then made alkaline by the addition of 0.75 ml of 0.1 M sodium borate (Sigma) before measurement of absorbance at 400 nm. Esterase activity was determined by reference to a standard curve of p-nitrophenol. A relative extracellular esterase (REE) unit was defined as the enzyme activity that liberates 1 nmol of *p*-nitrophenol from PNB in 1 h at 25°C at pH 5.5. Three replicate measurements of the extracellular esterase activity of all fungal isolates were made on separate occasions.

(iii) **pPVC weight loss.** Preweighed pPVC pieces (4.2 by 2.5 cm) were placed into petri dishes containing 30 ml of MSM liquid supplemented with 0.5 g of yeast extract $liter^{-1}$. Three replicate petri dishes were inoculated with 0.2 ml of spore or yeast suspension from each organism and incubated at 25°C for 6 weeks. Fungal biomass was removed from pPVC pieces by washing in nonionic detergent (LIP Lipsol, Shipley, United Kingdom), and samples were air-dried at room temperature (21 to 24°C) until a constant weight was reached. Statistically significant differences in percentage weight loss relative to control pPVC pieces incubated in sterile medium were determined using analysis of variance.

(iv) **Clear-zone production and colony growth on DOA agar.** Clear-zone production on agar plates containing emulsified DOA as the sole carbon source was used to test the ability of fungi to degrade DOA plasticizer. Plates containing 20 ml of DOA agar were inoculated with 50 μl of spore or yeast suspension placed into 5-mm-diameter wells cut at the center of each plate. Three replicate plates were inoculated for each organism and incubated at 25°C for 14 days. Clear-zone production on DOA-agar was scored according to the following criteria: 0, no clearing; 1, faint clearing below colony; 2, clearing extending beyond colony boundary; 3, intense clearing (agar completely transparent) extending beyond colony boundary. Colony growth was reported as follows: 0, no visible growth; 1, slight growth within inoculation well; 2, colony diameter < 2 cm; 3, colony diameter ≥ 2 cm. Tests for clear-zone production and growth on DOA agar were replicated on three separate occasions.

(v) **Growth using pPVC formulation as sole source of carbon.** pPVC pieces (4.2 by 2.5 cm) were placed on 15 ml of solidified MSM agar in a petri dish. A further 10 ml of molten MSM agar, cooled to 45°C, was inoculated with 0.1 ml

TABLE 1. Viable counts of fungi isolated from pPVC exposed in situ over 95 weeks

Sample wk (date)	All fungi on MEA (CFU cm^{-2})[a]	% DOA-degrading fungi[b]
10 (July 1997)	5 ± 1	74
25 (October 1997)	2 ± 0	65
40 (February 1998)	110 ± 27	84
55 (June 1998)	120 ± 15	76
80 (December 1998)	260 ± 27	71
95 (March 1999)	1,500 ± 220	80

[a] Mean viable counts ± 1 standard error of the mean from three replicate pPVC pieces, one taken from each in situ rack, are shown.

[b] Numbers of fungi recovered on DOA agar expressed as a percentage of all fungi recovered on MEA.

of spore or yeast suspension and poured over the pPVC. Plates were incubated at 25°C for 4 weeks. The following criteria were used to score growth on the pPVC: 0, no visible growth; 1, slight growth, barely visible; 2, growth clearly visible around the edges of the pPVC; 3, strong growth visible around edges and in the agar above the pPVC.

rDNA sequences. EMBL accession numbers for rDNA sequences from the 12 fungi identified in this study are as follows (sequences with which matches were made are shown in parentheses): MZ7, AJ276055 (UO5195); MZ8, AJ276054 (AJ000198); MZ10, AJ276058 (AIY17066); MZ14, AJ276057 (AJ005674); MZ20, AJ276059 (UO5915); MZ58, AJ276062 (AJ244236); MZ65, AJ276061 (AJ244236); MZ95, AJ276060 (AF138904); MZ103, AJ276063 (AF138289); MZ104, AJ276056 (AF033407); MZ107, AJ276065 (AF050278); MZ109, AJ276064 (U94948).

RESULTS

Viable counts of microorganisms colonizing pPVC in situ. Numbers of viable fungi, bacteria, and DOA-degrading organisms occurring on pPVC were monitored throughout the in situ trial (Table 1). Fungi were established on the pPVC surface after 40 weeks of exposure (February 1998; during United Kingdom's winter). No significant increase ($P = 0.82$) in fungal viable counts occurred during the next 15 weeks, but by week 80 (June 1998) the population had more than doubled. A rapid increase to 1,500 ± 220 CFU cm^{-2} (mean ± standard error) occurred in the last 15 weeks of exposure (December 1988 to March 1989; during United Kingdom's winter). CFU counts of fungi able to produce clear zones on DOA agar varied between 65 and 84% of the MEA fungal count.

Viable counts of fungi within the atmosphere. CFU counts were made on MEA and DOA agar plates exposed to the atmosphere for 20 h. The fungal counts (mean ± standard error) on plates of MEA and DOA agar were 40 ± 14 and 2 ± 1 CFU, respectively. Therefore, we estimate the proportion of DOA-degrading fungi deposited onto agar plates from the atmosphere was 5%.

Identification of in situ isolates. Fungi with 17 morphologically distinct colony types were identified. rDNA sequence lengths ranged from 496 to 576 bp for the 5.8S rDNA and ITS regions and from 518 to 562 bp for 28S rDNA. Representative colonies of 10 fungal morphotypes were identified using partial rDNA sequences, and these identifications were confirmed by microscopic examination and comparison with published descriptions of colony and conidial morphology. Unambiguous matches (98 to 100% sequence identity) were made using ITS sequences from isolates MZ8 (*Thanatephorous cucumeris*) (13), MZ10 (*Alternaria infectoria*) (13), MZ58 (*A. pullulans*) (13), MZ65 (*A. pullulans*), and MZ103 (*Emericella nidulans*; anamorph, *A. nidulans*) (13) and using the 28S V3 region from isolate MZ109 (*Taphrina deformans*) (10). The ITS sequence from isolate MZ104 showed 100% identity with *Penicillium glabrum* and 99.8% identity with *Penicillium lapidosum*, *Penicillium thomii*, and *Penicillium purpurescens*, differing from these species by only a single base pair. However, it was possible to distinguish MZ104 as *P. glabrum* on the basis of published colony morphology descriptions (33). ITS sequences from isolates MZ7 and MZ20 (both *Alternaria alternata*) showed the same level of identity (99.63%) with *Alternaria lini* as well as with *A. alternata*, and we were unable to differentiate these species following morphological examination. As *A. alternata* is an extremely common species found in abundance in soil and on other substrates (15), while *A. lini* is relatively rare (37) and may be contained within *A. alternata* (31), isolates MZ7 and MZ20 were named *A. alternata*. The ITS sequence of MZ14 had a relatively low (95%) identity with *Petromyces muricatus*. However, *P. muricatus* is a teleomorphic species of the *Aspergillus ochraceus* group (13, 41), and the colony and conidial morphology of MZ14 were identical to published descriptions of *A. ochraceus* (34). Thus MZ14 was named *Aspergillus ochraceus*. The 28S rDNA sequence from isolate MZ107 showed 95% identity with *Phaeococcomyces nigricans*. As species level identification requires further biochemical characterization, MZ107 was named only to the genus level.

Seven fungal morphotypes, including isolates MZ108 and MZ110 for which no ITS or 28S rDNA match was available, were identified at the International Mycological Institute using morphological techniques. Identification to the species level was possible for the yeasts MZ108 (*Kluyveromyces marxianus*) and MZ110 (*Rhodotorula aurantiaca*) and the filamentous fungi MZ111 (*Epicoccum nigrum*) and MZ112 (*Paecilomyces lilacinus*). MZ113 was identified only to the genus level (*Kluyveromyces* sp.), and MZ114, an intensely red yeast, could not be identified.

In situ colonization sequence. We made viable counts of the 17 fungal morphotypes throughout the colonization period (Table 2). *A. pullulans* was the primary colonizing isolate and was dominant between 25 and 80 weeks of exposure. After 25 weeks, *A. pullulans* was isolated from only two of the three in situ racks, but by 40 weeks this fungus was established on all three racks and its frequency had increased significantly (Table 2) ($P = 0.02$). The *A. pullulans* mean viable count was stable from 40 to 55 weeks ($P = 0.46$) but increased significantly from 55 to 80 weeks and again from weeks 80 to 95 (Table 2). After 95 weeks, larger colonies of *A. pullulans* were visible to the eye as black specks (≤2 mm in diameter) on the pPVC substratum.

A group of yeasts and yeast-like fungi were established after 80 weeks of exposure. These microorganisms had colony morphologies identical to isolates MZ107, MZ108, MZ109, MZ110, and MZ114. A significant increase in the numbers of each of these organisms occurred on all three racks between 80 and 95 weeks of exposure ($P \leq 0.005$) (Table 2). The most abundant of these isolates had the same colony morphology as MZ109. *E. nigrum*, a filamentous fungus, also appeared to be a secondary colonizer. It was initially isolated after 80 weeks from one of the in situ racks, but by 95 weeks this organism was recovered from all three racks (Table 2).

Throughout the in situ trial, several filamentous fungi and the yeast *K. marxianus* occurred sporadically (0 to 15 CFU cm^{-2}). The filamentous fungi included representatives of the genera *Alternaria*, *Aspergillus*, *Cladosporium*, *Emericella*, *Paecilomyces*, *Penicillium*, and *Thanatephorous*. Usually these organisms were isolated from only one or two of the in situ racks during sampling, although *Alternaria alternata* was recovered in low numbers from all three racks after 10 and 40 weeks of exposure (Table 2).

SEM of pPVC samples exposed in situ. pPVC samples exposed to the atmosphere for 95 weeks were examined under SEM (Fig. 1). Colonies of *A. pullulans*, 50 to 2,000 μm in

TABLE 2. Frequency of occurrence of fungi with different colony morphologies recovered from pPVC throughout the in situ trial

Colony morphotype	Mean CFU cm^{-2} at wk[a]					
	10	25	40	55	80	95
Aureobasidium pullulans	0	1 ± 1 (2)	93 ± 50 (3)	120 ± 35 (3)[b]	150 ± 20 (3)	390 ± 53 (3)
Epicoccum nigrum	0	0	0	0	0.3 ± 0.7 (1)	32 ± 13 (3)
Phaeococcomyces sp.	0	0	0	0	10 ± 9 (2)	64 ± 49 (3)
Pink-red yeast, unidentified	0	0	0	0	17 ± 11 (3)	170 ± 110 (3)
Rhodotorula aurantiaca	0	0	0	0	0.7 ± 0.7 (3)	73 ± 69 (3)
Taphrina deformans	0	0	0	0	86 ± 55 (3)	490 ± 240 (3)
Kluyveromyces sp.	0	0	0	0	0	220 ± 153 (3)
Alternaria alternata	3 ± 1 (3)	0.3 ± 0.6 (1)	4 ± 3 (3)	0	0	4 ± 7 (1)
Alternaria infectoria	0	0	0.2 ± 0.4 (1)	0	0.1 ± 0.3 (1)	0
Aspergillus niger	0.3 ± 0.5 (1)	0	0	0	0	7 ± 11 (2)
Aspergillus ochraceus	0	0	3 ± 5 (1)	0	0	0
Cladosporium herbarum	0	0	0	0	0	0.6 ± 0.7 (2)
Emericella nidulans	2 ± 2 (2)	0.3 ± 1.2 (1)[b]	0	0	0.1 ± 0.3 (1)	0
Kluyveromyces marxianus	0	0	0	0	0.6 ± 1.1 (1)	0
Paecilomyces lilacinus	0	0	0	0	0	6 ± 7 (2)
Penicillium glabrum	0.5 ± 1.2 (1)	0	12 ± 22 (1)	0	0	0
Thanatephorous cucumeris	0	0	0	0	0.3 ± 0.5 (2)	3 ± 5 (1)

[a] The mean viable counts and standard errors from three replicate pPVC pieces, one taken from each in situ rack, are shown. The number of racks on which fungi occurred at each sample time is indicated in parentheses.

[b] No statistically significant change in mean CFU count in comparison with previous sample time ($P > 0.05$).

diameter, were randomly dispersed across the surface of the pPVC. *A. pullulans* appeared both as young colonies in the early stages of development (Fig. 1a) and as well-established, circular or oval colonies with extensive hyphal growth (Fig. 1b). Yeast phase growth of *A. pullulans* was not observed, and mycelia appeared as chains of branching, septate hyphae. There was no evidence of penetration of the pPVC substratum by hyphae of *A. pullulans*. Very few other microorganisms were observed on the pPVC under SEM. Ovoid, yeast-like cells occurred occasionally either singly or in clumps of two to three cells, both associated with *A. pullulans* colonies and on uncolonized areas of the plastic. No bacteria were observed.

In vitro tests for biodeterioration of pPVC. Organisms with the highest level of extracellular esterase activity (100 to 250 REE ml^{-1}) included all three strains of *A. pullulans*, MZ10, MZ95, MZ107, and MZ111. Isolates with little or no extracellular esterase activity (≤2.0 REE ml^{-1}) included MZ110, MZ114, MZ103 (*Emericella nidulans*), and MZ8 (Table 3).

Extracellular esterase activity was not correlated with activity in other tests. No significant difference ($P = 0.15$) occurred between the mean esterase activity of isolates that produced strong DOA clear zones (score 3; MZ7, MZ10, MZ58, and MZ20) and those that demonstrated no clearing (score 0; MZ95, MZ104, MZ107, MZ108, MZ110, MZ114, and MZ115). Similarly, there was no significant difference ($P = 0.28$) in mean esterase activity between isolates showing strong growth on DOA agar (score 3; MZ7, MZ10, MZ14, MZ20, MZ95, MZ111, MZ112, and MZ115) and those showing no growth (score 0; MZ107, MZ108, MZ110, and MZ114). We also observed differences between strains of the same fungal species. For example, *A. alternata* strain MZ20 showed poor esterase activity in comparison with *A. alternata* strain MZ7. Measurements of extracellular esterase activity, clear-zone production, and growth on DOA agar were carried out on three separate occasions, and the same trends among all of the fungal isolates were observed.

The ability of in situ isolates to cause weight loss from pPVC was determined after incubation with pPVC for 6 weeks under MSM supplemented with yeast extract. The net weight loss in

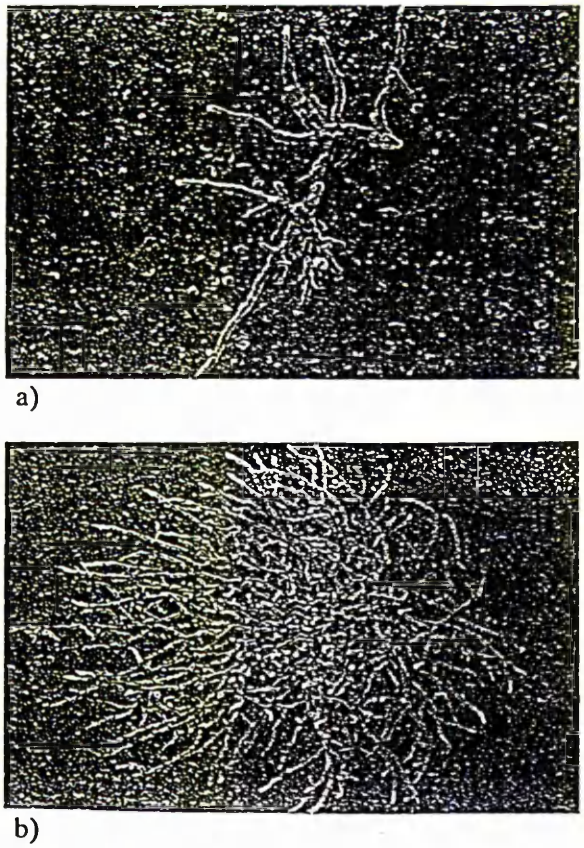

FIG. 1. SEM of the surface of pPVC exposed in situ for 95 weeks. (a) *A. pullulans* colony in early stages of development; (b) established *A. pullulans* colony.

TABLE 3. Deteriogenic properties of fungi isolated from pPVC during the in situ trial[a]

Isolate	Identification	Esterase activity (REE[b] ml^{-1})	Biomass in DOA liquid medium (mg/40 ml)	Specific esterase activity (REE mg^{-1})	Growth on DOA agar[c]	Clearing on DOA agar[d]	Growth on pPVC[e]	pPVC weight loss (%)[f]
MZ58	*Aureobasidium pullulans*	250 ± 40	11 ± 4.1	1500 ± 1300	1	3	1	3.7 ± 0.7
MZ10	*Alternaria infectoria*	240 ± 17	12 ± 0.3	800 ± 75	3	3	2	3.9 ± 0.5
MZ95	*Aspergillus niger*	190 ± 7	13 ± 2.8	630 ± 210	3	0	1	1.8 ± 0.2
PRA FS8	*Aureobasidium pullulans*	160 ± 6	8.8 ± 1.5	760 ± 240	1	2	3	3.6 ± 0.7
IMI70103	*Aureobasidium pullulans*	150 ± 16	5.2 ± 1.5	1300 ± 700	2	2	2	3.4 ± 0.7
MZ107	*Phaeococcomyces* sp.	120 ± 4	6.9 ± 2.4	840 ± 370	0	0	3	4.0 ± 0.2
MZ111	*Epicoccum nigrum*	100 ± 19	23 ± 4.2	180 ± 44	3	2	2	1.4 ± 0.3
MZ7	*Alternaria alternata*	81 ± 10	17 ± 0.4	300 ± 120	3	3	2	4.1 ± 0.3
MZ109	*Taphrina deformans*	68 ± 10	27 ± 9.6	160 ± 130	1	1	1	1.4 ± 0.4
MZ 113	*Kluyveromyces* sp.	51 ± 19	18 ± 12	260 ± 260	1	1	1	1.9 ± 0.7
MZ115	*Cladosporium herbarum*	50 ± 21	14 ± 4.2	150 ± 78	3	0	3	1.7 ± 0.4
MZ112	*Paecilomyces lilacinus*	27 ± 9	44 ± 7.0	26 ± 11	3	2	3	4.0 ± 1.5
MZ108	*Kluyveromyces marxianus*	18 ± 6	25 ± 8.7	49 ± 51	0	0	0	1.8 ± 0.2
MZ14	*Aspergillus ochraceus*	12 ± 5	17 ± 1.3	29 ± 8.4	3	2	1	3.9 ± 0.3
MZ20	*Alternaria alternata*	11 ± 6	12 ± 2.3	27 ± 14	3	3	2	4.2 ± 1.2
MZ104	*Penicillium glabrum*	5 ± 3	31 ± 5.3	8.1 ± 6.4	1	0	1	2.0 ± 0.4
MZ8	*Thanatephorous cucumeris*	2 ± 1	11 ± 0.6	6.1 ± 2.3	2	2	0	1.7 ± 0.1
MZ114	Unidentified pink-red yeast	0.4 ± 0.3	17 ± 3.6	1.3 ± 1.4	0	0	0	1.8 ± 0.4
MZ103	*Emericella nidulans*	0.4 ± 0.3	7.3 ± 3.2	6.5 ± 8.3	1	1	1	6.8 ± 0.6
MZ110	*Rhodotorula aurantiaca*	0.3 ± 0.4	16 ± 4.5	1.0 ± 1.5	0	0	0	1.9 ± 0.2

[a] Test methods used were measurement of extracellular esterase activity and biomass production during growth using DOA as the sole carbon source, clear-zone production, growth on DOA agar, growth on pPVC, and pPVC weight loss. Isolates are ranked in order of decreasing extracellular esterase activity. Mean values ± 1 standard error of the mean are shown.

[b] An REE is the enzyme activity that liberates 1 nmol of *p*-nitrophenol from PNB in 1 h at 25°C and at pH 5.5.

[c] Scores for growth on DOA agar: 0, no visible growth; 1, slight growth within inoculation well; 2, colony diameter < 2 cm; 3, colony diameter ≥ 2 cm.

[d] Scores for clear-zone production on DOA agar: 0, no clearing; 1, faint clearing below colony; 2, clearing extending beyond colony boundary; 3, intense clearing (agar completely transparent) extending beyond colony boundary.

[e] Scores for growth on pPVC: 0, no visible growth; 1, slight growth, barely visible; 2, growth clearly visible around the edges of the PVC; 3, strong growth visible around the edges and in the agar above the pPVC.

[f] The initial weight of individual pPVC pieces (100% weight loss value) ranged from 500 to 730 mg. The mean weight loss ± standard error of the mean from sterile pPVC controls was 0.1 ± 0.1%.

all cases was very low (7 to 50 mg), and therefore direct comparisons of the weight loss caused by individual species were not possible. However, all 20 isolates tested caused significant ($P \leq 0.01$) weight losses of >1% in comparison to control pPVC pieces incubated in sterile medium (Table 3). The greatest weight reduction, 6.8%, was caused by MZ103.

DISCUSSION

This study is the first detailed, quantitative investigation of the microbial colonization of pPVC. *A. pullulans* was the principal colonizing fungus. This organism initially colonized the pPVC after 25 weeks of exposure to the atmosphere and was isolated throughout the remainder of the in situ trial. SEM studies demonstrated that *A. pullulans* colonized pPVC in the absence of other microorganisms and therefore acts as a primary colonizer of pPVC. *A. pullulans* is increasingly recognized as the major causative agent of defacement of various diverse materials, such as painted surfaces (11, 21, 48) and wood (27), in addition to pPVC exposed to tropical conditions (24, 40). The present study demonstrates that *A. pullulans* is also an important colonizer of pPVC in temperate climates.

The success of *A. pullulans* in colonizing pPVC in situ is probably due to a combination of several factors. *A. pullulans*, which usually colonizes the phylloplane (13), can withstand periods of desiccation and high temperatures and produces highly melanized hyphae that protect against UV exposure (12). *A. pullulans* also produces extracellular polysaccharides that may facilitate permanent adhesion to surfaces (4), and factors controlling adhesion of *A. pullulans* to pPVC have recently been characterized (43). Therefore, adaptations for survival within the phylloplane probably confer advantages on *A. pullulans* for the colonization of painted and plastic surfaces within the environment (51).

A. pullulans also has considerable enzymatic capabilities. All three strains of *A. pullulans* produced high levels of extracellular esterase and could degrade DOA in vitro. In addition to producing extracellular esterase, *A. pullulans* also produces significant amounts of cellulase, proteinase, phosphatase, invertase, and maltase (47). The ability of *A. pullulans* to secrete such a variety of hydrolytic enzymes might enable it to utilize exogenous carbon sources that accumulate on the pPVC during long periods of exposure in situ. Extracellular esterase production is hypothesized to aid in the colonization of pPVC through the hydrolysis of organic-ester plasticizers (5, 28, 32). However, in this study extracellular esterase production did not correlate with DOA clearing or growth using pPVC as the sole source of carbon. These results may be due to differences in the specificity of esterase enzymes towards DOA plasticizers and the PNB synthetic substrate used in esterase assays. Thus, measurement of esterase activity alone is not a reliable indicator of the ability of an organism to degrade plasticizers or colonize pPVC.

A group of yeasts and yeast-like fungi became established on the pPVC much later than *A. pullulans*, towards the end of the in situ trial. Therefore, these yeasts probably play a secondary role in the colonization of pPVC in the sense that they require additional nutrients, e.g., the metabolites of other fungi or accumulated exogenous nutrients, before they can grow on the pPVC. These hypotheses are consistent with the observation that none of the yeasts, except *Kluyveromyces* sp., can degrade DOA or grow on pPVC as the sole source of carbon in vitro. Yeasts are not generally considered as important deteriogenic organisms on artificial surfaces, even though they have been

recovered from deteriorated rubber and building materials (29) and from deteriorated pPVC during tropical exposure trials in Puerto Rico (24). Neither study provided information on the abundance of the yeasts or their role in the biodeterioration of these materials. Thus, the present study is the first to attribute a significant role to yeasts in the colonization of pPVC.

Interestingly, only a few yeast cells were observed during SEM studies of the pPVC substratum. These results suggest that high yeast CFU counts resulted from a small number of rapidly multiplying yeast colonies and highlight the general problem of using CFU counts to quantify fungi. CFU counts depend on how readily the fungal material on the pPVC breaks into individual propagules during the isolation procedure. For example, for the same amount of biomass, a colony of a budding yeast or a sporulating filamentous fungus may yield many more CFUs than a spreading hyphal mycelium. Thus, while CFU counts are useful in determining which organisms are colonizing and multiplying on the pPVC, they are not usually a reliable indicator of the fungal biomass present on the substratum.

Bacteria were not isolated from the pPVC during the trial, and none were detected during SEM studies or following DAPI (4',6'-diamidino-2-phenylindole) fluorescence staining of organisms removed from the pPVC substratum (data not shown). Bacterial growth might be inhibited by desiccation, solar irradiation, or in situ acidification of the pPVC following photochemical or thermal degradation (25, 30). Acidification of the pPVC could inhibit bacterial growth, as fungi tolerate lower pHs than do bacteria.

Fungal colonization of pPVC appeared to be influenced by seasonal climatic changes. Major increases in fungal CFU counts occurred only during the British winter. For example, fungal CFU counts increased by 470% during the winter period between November 1998 (80 weeks) and March 1999 (95 weeks), largely due to the establishment of yeasts and yeast-like fungi on the pPVC. In contrast, no increases in fungal CFU counts occurred when pPVC pieces were sampled during the British summer months. Fungal growth or sporulation during the summer period might be inhibited by desiccation and high temperatures caused by long periods of direct solar irradiation. Indeed, Upsher and Roseblade (40) reported that a marked reduction in the amount of fungal growth on pPVC can occur during dry periods. Extended studies of the colonization process are needed to determine whether yeasts that establish on the pPVC during the winter months can survive on the pPVC through the succeeding summer period.

Whether an organism can colonize pPVC also probably depends on its ability to obtain carbon from the pPVC formulation. Evidence supporting this hypothesis comes from the observation that between 64 and 84% of fungi colonizing pPVC could degrade DOA. In contrast, among fungi deposited onto agar plates from the atmosphere, only 5% could degrade DOA at the time of sampling. Although the proportion of DOA-degrading fungi within the atmosphere is likely to be influenced by environmental parameters and probably varies seasonally, these results support the hypotheses that selection for fungi that can degrade DOA occurs on the pPVC substratum and that organisms that can degrade DOA may have a competitive advantage during the colonization of pPVC.

In addition to *A. pullulans*, many of the recovered fungi, e.g., *Alternaria* spp., *Aspergillus* spp., *Paecilomyces* sp., and *Cladosporium* sp., have previously been isolated from deteriorated pPVC in tropical exposure trials (24, 36) and are common colonizers of painted surfaces and building materials (for a review, see reference 19). In particular, *A. alternata*, *A. infectoria*, and *P. lilacinus* had high activity in all of the test methods, demonstrating that they are potentially important degraders of pPVC within the environment. Under ideal growth conditions within warm and humid tropical exposure trials, these organisms would probably grow readily on the pPVC substratum. However, these organisms were isolated infrequently and in low numbers in this study, suggesting that environmental factors limited the establishment of these organisms on the pPVC. Thus, while in vitro methods identified fungi potentially capable of causing biodeterioration of pPVC, they were not predictive of the organisms that colonized pPVC in the environment.

We observed discrepancies between the ability of fungi to grow on the intact pPVC formulation as the sole carbon source and their ability to degrade DOA in vitro. For example, *Phaeococcomyces* sp. grew well on pPVC but could not produce clear zones in DOA agar. It is possible that components of the pPVC formulation other than the plasticizers can support the growth of fungi on pPVC. We used DOA in the present study because it is known to be more susceptible than the other plasticizer, DOP, to microbial attack (5). However, in addition to DOA and DOP the pPVC formulation also contains small quantities of a calcium-zinc stearate stabilizer and an epoxidized oleate ester stabilizer that can be utilized by various fungi as a sole carbon source (36). Thus, while the homogenized plasticizer agar technique is useful to determine if an organism can degrade a plasticizer, it is not necessarily predictive of the organism's ability to grow on a complex pPVC formulation.

We found no evidence that the mechanical properties of the pPVC were altered due to microbial degradation of plasticizers in the environment. Although fungi can increase the tensile strength of pPVC in vitro due to the degradation of plasticizers (39, 46), tensile testing of exposed pPVC pieces showed no significant change in either the tensile strength or the percent elongation at breaking (data not shown). We think that the fungal biomass that accumulated on pPVC pieces during the in situ trial was insufficient to cause a measurable change in the mechanical properties of the pPVC.

In summary, our results suggest that a colonization sequence may occur during colonization of pPVC in situ. *A. pullulans* is the principal colonizing fungus, and secondary colonizing yeasts establish themselves much later. In vitro biodeterioration tests were not predictive of the ability of fungi to colonize pPVC in the environment, emphasizing the importance of field trials in investigations of the microbial susceptibility of pPVC formulations. Knowledge of the organisms that colonize pPVC and their ecology is essential for the design of novel pPVC formulations and biocides that provide long-term protection against biodeterioration of pPVC in situ.

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