Signalling and Regulation in *Candida* biofilms

By

Mohammed A. S. Alem (MSc)

Presented for the degree of Doctor of Philosophy
University of Glasgow

Division of Infection and Immunity,
Institute of Biomedical and Life Sciences
University of Glasgow

JULY 2006

PREFACE

This thesis is the original work of the author, apart from tyrosol measurement by HPLC which was done in collaboration with Mohammed Oteef (PhD student), Department of Chemistry, University of Glasgow.

Mohammed A. S. Alem

DEDICATION

This is dedicated to my wife "Mrs. Anan Rammadan" for her appropriate enthusiastic participation through my academic career, my two wonderful children Hala and Hamad, my family and to the memory of my father.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank Dr. L.J. Douglas for her supervision, phenomenal advice and encouragement throughout this study.

I am indebted to all staff, technicians, students on the Division of Infection and Immunity, especially Mrs. Susan Baillie for her friendliness. Thus I have never known her to refuse to offer a lending hand and she always is at ringside.

I am indebted to Margaret Mullin for expert assistance with electron microscopy.

I am indebted to Mohammed Oteef (PhD student) and Dr. Hugh Flowers, Department of Chemistry, University of Glasgow, for expert assistance to measure tyrosol by HPLC.

Finally, thanks go out to Ministry of Health, Saudi Arabia, for this scholarship opportunity, and friends, relatives, and supporters who have made this happen.

CONTENTS

PREFACE ii
DEDICATION iii
ACKNOWLEDGEMENTS iv
CONTENTS v
LIST OF TABLES xiii
LIST OF FIGURES xv
ABBREVIATIONS xix
SUMMARY xxi

INTRODUCTION

1. *Candida* and Candidosis **1**
1.1 *Candida* species 1
1.2 *Candida* infections 1
1.2.1 Superficial candidosis 2
1.2.2 Systemic candidosis 3
1.2.3 Implant-associated *Candida* infections 4
1.3 Predisposing factors for *Candida* infection 5
1.4 Treatment and antifungal agents 6
2. Virulence factors of *Candida* species **8**
2.1 Adhesion 8
2.1.1 Adhesins and host cell receptors 9
2.2 Dimorphism 11
2.3 Production of extracellular enzymes 12
2.4 *C. albicans* transcription factors 14
3. Microbial biofilms **15**
4. *Candida* biofilms **17**
4.1 Biofilm model systems 17
4.2 Factors affecting the ability of *Candida* to form biofilms 18
4.3 Biofilm ultrastructure 19
4.3.1 Mixed fungal-bacterial biofilms 22
4.4 Drug resistance of biofilms 23
4.5 Possible mechanisms of biofilm drug resistance 24
4.5.1 Restricted drug penetration through biofilm matrix material 24
4.5.2 Slow growth rate 25

4.5.3 Differential gene expression 26
4.5.4 "Persister" cells 27
5. Quorum sensing 28
5.1 Quorum sensing in *Candida* by farnesol 30
5.1.1 Farnesol and biofilms 34
5.1.2 Quorum sensing in fungal-bacterial biofilms 35
5.2 Quorum sensing in *Candida* by tyrosol 37
6. Hormones and *Candida* 38
6.1 Prostaglandins 38
6.2 COX inhibitors 40
6.2.1 COX inhibitors and antibacterial drugs 42
6.2.2 COX inhibitors and antifungal drugs 43
6.3 Steroid hormones 44
Aims and Objectives of Research 46

MATERIALS AND METHODS

1. Organisms 48
2. Growth Media 48
2.1 Yeast nitrogen base 48
2.2 Yeast nitrogen base plus uridine 48
2.3 Yeast nitrogen base plus 17 mM acetic acid 48
2.4 Sabouraud dextrose agar 51
2.5 Sabouraud dextrose broth 51
2.6 RPMI 1640 buffered with MOPS 51
2.7 RPMI 1640 buffered with HEPES 51
2.8 Yeast peptone dextrose 52
2.9 Vagina-simulative medium 52
2.10 Hornby medium 52
2.11 Germ-tube inducers 52
3. Chemicals 53
3.1 COX inhibitors and PGE_2 53
3.2 Steroids 53
3.3 Quorum sensing molecules 53
3.3.1 Farnesol 53

3.3.2 Tyrosol 54
3.4 Propranolol 54
3.5 1-Oleoyl-2-acetyl-sn-glycerol (OAG) 54
3.6 Dibutyryl cyclic-AMP 54
3.7 Antifungal agents 54
3.7.1 Amphotericin B 54
3.7.2 Fluconazole 54
4. Biofilm formation on catheter disks 55
4.1 Preparation of catheter disks 55
4.2 Preparation of microorganisms 55
4.3 Biofilm formation 57
4.4 Quantitative measurement of biofilm formation 57
5. Biofilm formation in wells of microtitre plates 58
6. Measurement of tyrosol in culture supernatants 59
6.1 Supernatant preparation 59
6.1.1 Growth of planktonic cells and biofilms 59
6.1.2 Dry weight determinations 59
6.2 Tyrosol extraction and quantification using HPLC 60
6.2.1 Chemicals 60
6.2.2 Solid phase extraction (SPE) method 60
6.2.3 HPLC equipment 62
6.2.4 Chromatographic conditions 62
6.2.5 Method validation 64
7. Germ-tube formation 64
7.1 Measurement of germ-tube formation 64
7.2 Effect of biofilm supernatant or farnesol on planktonic cell morphology 67
7.3 Effect of planktonic cell and biofilm supernatant on germ-tube formation 67
7.4 Germ-tube formation in the presence of COX inhibitors 68
8. Scanning electron microscopy 69
8.1 Air-drying method 69
8.2 Freeze-drying method 69
9. Antifungal susceptibility testing by the NCCLS M27 method 70
9.1 Inoculum preparation 70

9.2 Microdilution method 70
9.3 Macrodilution method 71
10. Prostaglandin detection by ELISA 71
10.1 Preparation of samples 71
10.2 ELISA plate assay 72
10.3 ELISA plate development and reading 72
11. Determination of cell dry weight 74
12. Statistical analysis of data 74

RESULTS

1. Preliminary studies on biofilms and germ-tube development in *C. albicans* 75
1.1 Quantitative measurement of biofilm formation using the XTT assay ... 75
1.2 Determination of optimal glucose concentration for the XTT assay 75
1.3 Standard curve of planktonic cell count against OD $_{520}$ reading 78
1.4 Biofilm formation by *C. albicans* strains obtained from the University of Nebraska. 78
1.5 Biofilm formation by *C. albicans* strains obtained from Columbia University, New York 78
1.6 Biofilm formation by *C. albicans* SC 5314 and its morphological mutants 79
1.7 Germ-tube formation with different inducers 84
1.8 Germ-tube formation in the presence of farnesol 84
1.9 Effect of propranolol and 1-oleoyl-2-acetyl-sn-glycerol (OAG) on germ-tube formation by *C. albicans* GDH 2346 85
2. Role of farnesol as a quorum sensing molecule in *C. albicans* biofilms 91
2.1 Biofilm formation by *C. albicans* GDH 2346 in the presence of farnesol 91
2.2 Effect of 1 mM farnesol on biofilm formation by *C. albicans* GDH2346 and *C. albicans* CAI-4 grown on Hornby medium or RPMI 1640 93
2.3 Biofilm formation by *C. albicans* GDH 2346 in the presence of farnesol and 1-oleoyl-2-acetyl-sn-glycerol (OAG) 95
2.4 Scanning electron microscopy of the effects of farnesol, propranolol and OAG on *C. albicans* GDH 2346 biofilms grown in YNB, RPMI 1640 and Hornby medium 95
2.5 Effect of farnesol on planktonic inocula for biofilm formation by different *C. albicans* strains 99
2.6 Biofilm formation in microtitre plates in the presence of farnesol 102

2.7 Effect of different farnesol concentrations on biofilm formation by *C. albicans* strains obtained from the University of Nebraska 104

2.8 Scanning electron microscopy of biofilm formation by *C. albicans* Nebraska strains in the presence of 1 mM farnesol 106

2.9 Effect of biofilm culture supernatants on the morphology of planktonic cells 109

2.10 Effect of planktonic and biofilm culture supernatants on germ-tube formation by *C. albicans* 112

2.11 Biofilm formation by morphological mutants in the presence of 1 mM farnesol or propranolol 114

2.12 Scanning electron microscopy of biofilm formation by *C. albicans* SC5314 and its morphological mutants in the presence of farnesol or propranolol 114

2.13 Biofilm formation by *C. albicans* CAF2-1 and its quorum sensing mutant strains lacking *CHK1* 115

3. Role of tyrosol as a quorum-sensing molecule in *C. albicans* biofilms 119

3.1 Biofilm formation in the presence of tyrosol 119

3.2 Scanning electron microscopy of the effect of tyrosol on biofilm formation by *C. albicans* GDH 2346 122

3.3 Scanning electron microscopy of the effect of tyrosol on biofilm formation by *C. albicans* SC5314 and its morphological mutants 122

3.4 Tyrosol production by planktonic cells of *C. albicans* 125

3.5 Tyrosol production by biofilms of *C. albicans* 125

3.6 Comparison of tyrosol production by planktonic cells and biofilms on the basis of cell dry weight 126

3.7 Effect of simultaneous addition of farnesol and tyrosol on biofilm formation 130

3.8 Scanning electron microscopy of biofilm formation in the presence of farnesol and/or tyrosol 130

3.9 Scanning electron microscopy of the effect of tyrosol on the early stages of biofilm formation by *C. albicans* 131

4. Effects of aspirin and other nonsteroidal anti-inflammatory drugs on biofilm and planktonic cells of *C. albicans* 135

4.1 Effect of PGE_2 on biofilm formation by *C. albicans* 135

4.2 Effect of COX inhibitors on biofilm formation 136

4.3 Inhibition of biofilm formation by different concentrations of aspirin 142

4.4 Aspirin addition during different stages of biofilm growth 142

4.5 Aspirin addition to mature biofilms 143

4.6 Effect of simultaneous addition of aspirin and PGE_2 on biofilm formation 148

4.7 Viability of biofilms after treatment with COX inhibitors 148

4.8 Scanning electron microscopy of biofilms exposed to COX inhibitors . 151

4.9 Effect of COX inhibitors on germ-tube formation 151

4.10 Effect of simultaneous addition of aspirin and cAMP on biofilm formation by *C. albicans* GDH 2346 152

5. Combined effects of COX inhibitors and antifungal agents on *Candida* biofilms 157

5.1 MICs of aspirin and / or amphotericin B for planktonic *C. albicans* GDH 2346 157

5.2 Effect of aspirin and amphotericin B on biofilm formation by *C. albicans* GDH 2346 158

5.3 Effect of aspirin, salicylic acid and amphotericin B on biofilm formation by *C. albicans* GDH 2346 in different media 160

5.4 Effect of COX inhibitors other than aspirin on biofilm formation by three *C. albicans* strains grown in two different media 160

5.5 Relationship between optical density and viability of planktonic cells of *C. albicans* GDH 2346 grown in buffered and unbuffered YNB in the presence of COX inhibitors 164

5.6 Effect of aspirin on the optical density and viability of planktonic cells of *C. albicans* GDH 2346 grown in different buffered media 169

5.7 Relationship between the effect of 1 mM aspirin on optical density and the pH value of the growth medium for planktonic cells 170

5.8 Viable counts of planktonic and biofilm cells of *C. albicans* GDH 2346 grown at different pH values with 1 mM aspirin 170

5.9 Effects of COX inhibitors on metabolic activity of mature biofilms of *C. albicans* GDH 2346 grown in unbuffered YNB and YNB at pH 7 ... 175

5.10 Effects of 1 mM aspirin on viability and metabolic activity of mature (48-h) *C. albicans* GDH 2346 biofilms grown in YNB buffered at different pH values 177

5.11 Effect of aspirin and fluconazole on metabolic activity of mature (48-h) *C. albicans* GDH 2346 biofilms grown at different pH values 177

5.12 Effects of aspirin and fluconazole on metabolic activity of mature (48-h) biofilms of *C. albicans* GDH 2346 grown in three different media 180

5.13 Effects of aspirin and fluconazole on metabolic activity of mature (48-h) biofilms of *C. glabrata* AAHB 12 grown in three media 180

5.14 Viability of mature (48-h) biofilms of *C. albicans* GDH 2346 and *C. glabrata* AAHB 12 after treatment with 200 µM or 1 mM aspirin 181

6. Prostaglandin production during growth of *C. albicans* biofilms 185

6.1 Development of prostaglandin standard curve using growth medium ... 185

6.2 Prostaglandin production by biofilms and planktonic cells of *C. albicans* GDH 2346 186

6.3 Effects of COX inhibitors on prostaglandin production by biofilms and planktonic cells of *C. albicans* GDH 2346 189

6.4 Prostaglandin production by *C. albicans* SC5314 and its morphological mutant HLC54 (*cph1/cph1 efg1/efg1*) 189

6.5 Scanning electron microscopy of *C. albicans* SC5314 and its morphological mutant HLC54 (*cph1/cph1 efg1/efg1*) 190

7. Effects of steroids on biofilm development and germ-tube formation by *C. albicans* 194

7.1 Biofilm formation in the presence of steroids 194

7.2 Effect of steroids on germ-tube formation by *C. albicans* 194

DISCUSSION

1. Biofilm development and germ-tube formation in *C. albicans* 198

1.1 Biofilm formation using different *C. albicans* strains 198

1.2 Germ-tube formation in *C. albicans* 200

2. Quorum sensing in *C. albicans* biofilms 202

2.1 The role of farnesol 202

2.2 The role of tyrosol 208

3. Effect of COX inhibitors on *Candida* biofilms 212

4. Combined effect of COX inhibitors and antifungal agents on *Candida* 215

5. Prostaglandin production in planktonic cells and biofilms of *C. albicans* .. 220

6. Biofilm formation in the presence of steroids 222

7. Overall conclusions 225

REFERENCE 227

APPENDICES

Appendix 1. Composition of Growth Media 261

App. 1.1 Yeast Nitrogen Base (YNB; Difco) 261

App. 1.2 Sabouraud Dextrose Broth (SDB;Oxoid) 261

App. 1.3 Sabouraud Dextrose Agar (SDA;Oxoid) 262

App. 1.4 Vagina-simulative medium (VS) 262

App. 1.5 RPMI 1640 with HEPES (Sigma) 262
App. 1.6 RPMI 1640 with L-Glutamine (Cambrex)..... 263
App. 1.7 Yeast peptone dextrose (YPD) medium 265
App. 1.8 Hornby medium 265
App. 1.9 Germ-tube inducers..... 266
Appendix 2. buffers..... 266
App. 2.1 0.01 M phosphate buffered saline pH 7.2 ± 0.2 (Sigma)..... 266
App. 2.2 0.1M citric acid – 0.2M disodium hydrogen phosphate buffer 267
App. 2.3 Tris maleate – sodium hydroxide buffer (pH 8) 267
App. 2.4 1 M phosphate buffer..... 268
App. 2.5 (10x) EIA (electroimmunoassay) buffer..... 268
Publications and poster abstracts arising from this work..... 269

LIST OF TABLES

Table 1. Organisms used in this study 49

Table 2. ELISA plate pipetting summary 73

Table 3. Germ-tube formation by *C. albicans* SC5314 and its morphological mutants 90

Table 4. Biofilm formation of *C. albicans* SC5314 and its morphological mutants with farnesol and propranolol 116

Table 5. Effect of PGE_2 on germ-tube formation by two *C. albicans* strains..... 137

Table 6. Biofilm formation by *C. albicans* GDH 2346 in the presence of PGE_2 138

Table 7. Effects of COX inhibitors on biofilm formation by three strains of *C. albicans* 139

Table 8. Effect of salicylic acid on biofilm formation by three strains of *C. albicans* 141

Table 9. Effect of aspirin addition at different times during biofilm formation by *C. albicans* SC5314..... 145

Table 10. Effect of aspirin on XTT reduction by mature (48-h) biofilms of *C. albicans* GDH 2346 146

Table 11. Effect of aspirin on viability of mature (48-h) biofilms of *C. albicans* GDH 2346 147

Table 12. Effect of simultaneous addition of aspirin and PGE_2 on biofilm formation by *C. albicans* GDH 2346 149

Table 13. Viability of planktonic and biofilm cells of *C. albicans* GDH 2346 after growth for 24 h in the presence of different COX inhibitors [a]..... 150

Table 14. Effect of simultaneous addition of aspirin and CAMP on biofilm formation by *C. albicans* GDH 2346 156

Table 15. Effect of aspirin and amphotericin B on biofilm formation by *C. albicans* GDH 2346 in YNB (with and without MOPS) 159

Table 16. Effect of aspirin, salicylic acid and amphotericin B (AmB) on biofilm formation by *C. albicans* GDH 2346 in different media 162

Table 17. Biofilm formation by three strains of *C. albicans* with COX inhibitors in two different media 163

Table 18. pH measurements during planktonic growth of *C. albicans* GDH 2346 for 24h in YNB, with or without MOPS, and 1 mM COX inhibitor..... 168

Table 19. Effect of COX inhibitors on XTT reduction by mature (48-h) biofilms of *C. albicans* GDH 2346 176

Table 20. Viability of mature (48-h) biofilms of *C. albicans* GDH 2346 and *C. glabrata* AAHB 12 after treatment with 200 μM or 1 mM aspirin 184

Table 21. Effects of COX inhibitors on prostaglandin production by biofilms and planktonic cells of *C. albicans* GDH 2346 191

Table 22. Effect of steroids on germ-tube formation by *C. albicans* GDH 2346 197

LIST OF FIGURES

Figure 1 Stages in the formation of a *C. albicans* biofilm 21

Figure 2 Quorum sensing molecules in *C. albicans* 32

Figure 3 Synthases of Prostaglandins 39

Figure 4 Structures of COX inhibitors 41

Figure 5 Biofilm disks and metal punch 56

Figure 6 Sep-Pak cartridge and vacuum manifold 61

Figure 7 HPLC system used for the separation and quantification of tyrosol 63

Figure 8 Tyrosol calibration curve 65

Figure 9 Typical HPLC chromatograms 66

Figure 10 Quantitative measurement of biofilm formation by *C. albicans* GDH 2346 using the XTT assay 76

Figure 11 Effect of different glucose concentrations in the XTT assay for measurement of biofilm formation by *C. albicans* GDH 2346 77

Figure 12 Standard curve of planktonic cell count against OD_{520} reading for *C. albicans* GDH 2346 80

Figure 13 Biofilm formation by *C. albicans* strains obtained from the University of Nebraska 81

Figure 14 Biofilm formation by *C. albicans* strains obtained from the Columbia University, New York 82

Figure 15 Biofilm formation by *C. albicans* SC 5314 and its morphological mutants 83

Figure 16 Germ-tube formation by *C. albicans* GDH 2346 in the presence of different inducers 87

Figure 17 Germ-tube by formation of different *C. albicans* strains 88

Figure 18 Effects of farnesol, propranolol and OAG on germ-tube formation by *C. albicans* GDH 2346 89

Figure 19 Effect of farnesol concentration on biofilm formation of *C. albicans* GDH 2346 grown in YNB medium 92

Figure 20 Effect of farnesol on *C. albicans* biofilms grown in Hornby medium or RPMI 1640 medium 94

Figure 21 Effect of farnesol and /or OAG on biofilm formation by *C. albicans* GDH 2346 97

Figure 22 Scanning electron micrographs of the effects of farnesol, propranolol and OAG on biofilms of *C. albicans* GDH 2346 grown in three different media 98

Figure 23 Effects of 1 mM farnesol on overnight cultures and biofilm formation by different *C. albicans* strains grown in Hornby medium 100

Figure 24 Effect of 1 mM farnesol on overnight cultures and biofilm formation of *C. albicans* GDH 2346 grown in YNB medium 101

Figure 25 Effect of farnesol on biofilm formation in wells of microtitre plates. 103

Figure 26 Effect of three different farnesol concentrations on biofilm formation by *C. albicans* mutants obtained from the University of Nebraska 105

Figure 27 Scanning electron micrographs by biofilm formation by *C. albicans* mutants obtained from the University Nebraska 107

Figure 28 Effect of biofilms supernatant on hypha formation of planktonic cells growth by different *C. albicans* strains 110

Figure 29 Effect of different biofilm supernatants on planktonic cells of *C. albicans* CAI-4 111

Figure 30 Effects of biofilm and planktonic culture supernatants on germ- tube formation by *C. albicans* SC5314 113

Figure 31 Scanning electron micrographs of biofilm formation by *C. albicans* SC5314 and its morphological mutants in the presence of farnesol and propranolol 117

Figure 32 Biofilm formation by *C. albicans* CAF2-1 and its quorum sensing mutant strains lacking Chk1p 118

Figure 33 Effect of tyrosol on biofilm formation by *C. albicans* GDH 2346 120

Figure 34 Effect of different tyrosol concentrations on biofilm formation by *C. albicans* GDH 2346 grown in YNB or RPMI 1640 medium 121

Figure 35 Scanning electron micrographs showing the effect of tyrosol on *C. albicans* GDH 2346 biofilms grown in YNB. 123

Figure 36 Effect of tyrosol on biofilm structure of *C. albicans* SC5314 and its morphological mutants 124

Figure 37 Relationship between tyrosol productions and planktonic cell dry weight of *C. albicans*. 127

Figure 38 Relationship between tyrosol production and biofilm cell dry weight 128

Figure 39 Tyrosol production expressed as a function of cell dry weight for planktonic cells and biofilms. 129

Figure 40 Effect of simultaneous addition of farnesol and tyrosol on biofilm formation by *C. albicans* GDH 2346 132

Figure 41 Scanning electron micrographs of *C. albicans* SC5314 biofilms grown on polystyrene disks in the presence of farnesol and tyrosol 133

Figure 42 Scanning electron micrographs showing the effect of 50 μM tyrosol on the early stages of biofilm formation by *C. albicans* 134

Figure 43 Effect of 1 mM aspirin on biofilm formation by different *C. albicans* strains 140

Figure 44 Inhibition of biofilm formation by *C. albicans* GDH 2346 by different concentrations of aspirin 144

Figure 45 Scanning electron micrographs of *C. albicans* GDH 2346 biofilms grown in the presence of aspirin 153

Figure 46 Scanning electron micrographs of *C. albicans* GDH 2346 biofilms grown in the presence of different COX inhibitors 154

Figure 47 Effects of COX inhibitors (final concentration 100 μM) on germ-tube formation by *C. albicans* GDH 2346 155

Figure 48 Relationship between optical density and viability of planktonic cells of *C. albicans* GDH 2346 grown in unbuffered YNB with different COX inhibitors present at 1 mM 166

Figure 49 Relationship between optical density and viability of planktonic cells of *C. albicans* GDH 2346 grown in YNB+MOPS (pH 7) with different COX inhibitors present at 1 mM 167

Figure 50 Effect of aspirin on the optical density and viability of planktonic cells of *C. albicans* GDH 2346 grown in different buffered media 172

Figure 51 Effect of 1 mM aspirin on the optical density of *C. albicans* GDH 2346 cultures grown at different pH values 173

Figure 52 Viable counts of planktonic and biofilm cells of *C. albicans* grown at different pH values with 1 mM aspirin 174

Figure 53 Effects of 1 mM aspirin on viability and metabolic activity of mature (48-h) biofilms of *C. albicans* GDH 2346 grown at different pH values 178

Figure 54 Effects of aspirin and fluconazole on metabolic activity of mature (48-h) biofilms of *C. albicans* GDH 2346 grown at different pH values 179

Figure 55 Effects of aspirin and fluconazole on metabolic activity of mature (48-h) biofilms of *C. albicans* GDH 2346 grown in three different media ... 182

Figure 56 Effects of aspirin and fluconazole on metabolic activity of mature (48-h) biofilms of *C. glabrata* AAHB 12 grown in three media ... 183

Figure 57 Prostaglandin standard cure for the prostaglandin screening kit ... 187

Figure 58 Prostaglandin production by biofilms and planktonic cells of *C. albicans* GDH 2346 ... 188

Figure 59 Prostaglandin production by *C. albicans* SC5314 and its morphological mutant HLC54 (*cph1/cph1 efg1/efg1*) ... 192

Figure 60 Scanning electron microscopy of *C. albicans* SC5314 and its morphological mutant HLC54 (*cph1/cph1 efg1/efg1*) ... 193

Figure 61 Effect of steroids on biofilm formation by *C. albicans* GDH 2346 ... 196

Figure 62 Operation of different signalling pathways during morphogenesis and biofilm formation in *C. albicans* ... 226

ABBREVIATIONS

A_{492}	Absorbance at 492nm
Ad	Adhesion
AHL	N-Acylhomoserine lactone
AmB	Amphotericin B
AMP	Adenosine monophosphate
aq	Aqueous
AU	Absorbance unit
cAMP	Adenosine cyclic monophosphate
COX	Cyclooxygenase isoenzymes
DGA	Diacylglycerol
DMSO	Dimethyl sulfoxide
EIA	Buffer (appendix 2.5)
ELISA	Enzyme-linked immunoabsorbent assay
Expt	Experiment
5FC	5-Fluorocytosine
GDH	Glasgow Dental Hospital, Scotland UK
GlcNAc	N-acetylglucosamine
GPP	Glucose phosphate proline medium
HEPES	4-2-Hydroxyethyl-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
IgG	Immunoglobulin G
MIC	Minimum inhibitory concentration
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	Messenger ribonucleic acid
NCCLS	National Committee for Clinical Laboratory Standards
ND	Not determined
OD	Optical density
OAG	1-Oleoyl-2-Acetyl-sn-Glycerol
P	Probability value
PBS	Phosphate-buffered saline

pg	Picogram
PG	Prostaglandin
pKa	negative log to base 10 of the acidity constant
PLD	Phospholipase D
Pro	Proline
Prop	Propranolol
p.s.i	Pounds per square inch
PVC	Polyvinyl chloride
RNA	Ribonucleic acid
RP-HPLC	Reverse phase-high performance liquid chromatography
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
SEM	Standard error of the mean
SPE	Solid phase extraction
UV	Ultraviolet
VS	Vagina-simulative medium
XTT	2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
YNB	Yeast nitrogen base
YPD	Yeast peptone dextrose

SUMMARY

Candida albicans and related *Candida* species are common members of the normal flora in humans; however, they are opportunistic pathogens and may cause superficial or systemic disease. Candidaemia is the most extensively studied nosocomial invasive fungal infection, and recent data show that *Candida* species are the fourth most commonly recovered blood culture isolates. It is now clear that this kind of infection is often due to the formation of *Candida* biofilms on catheters and other indwelling medical devices. This project investigates the role of quorum sensing in *Candida* biofilm formation, and the effect of prostaglandins, COX inhibitors and the combined effect of COX inhibitors with antifungal agents on biofilm development. Additional investigations monitor prostaglandin production by planktonic cells and biofilms of *Candida albicans*.

Quorum sensing is known to be involved in bacterial biofilm formation, and in *Candida*, two signal molecules (farnesol and tyrosol) were recently identified. In this study the effects of farnesol on germ-tube and biofilm formation were investigated. Farnesol totally prevented germ-tube formation and inhibited biofilm formation by 30 % when 1mM farnesol was added at an early stage. Scanning electron microscopy revealed that only yeast cells were formed in biofilms grown in the presence of farnesol. Supernatants from biofilms formed after 24h and 48h inhibited germ-tube formation by 10 % and 29 %, respectively, indicating that they contained farnesol or farnesol-like activity.

It has been reported that planktonic cultures of *C. albicans* produce tyrosol, another quorum-sensing molecule. In this study, HPLC confirmed that tyrosol was produced by both planktonic cells and biofilms of *C. albicans*, and biofilms produced significantly higher levels of tyrosol than planktonic cells. Overall, the results demonstrated that biofilm development in *C. albicans* is under the control of at least two quorum-sensing molecules. Farnesol acts as a negative signal and inhibits hyphal production. Tyrosol acts as a positive signal and promotes hyphal production.

Prostaglandins are now known to be produced by *C. albicans* and may play an important role in fungal colonization. Their synthesis in mammalian cells is decreased by inhibitors of the cyclooxygenase isoenzymes required for prostaglandin formation. In this study, the effects of nonsteroidal anti-inflammatory drugs (all cyclooxygenase inhibitors) on biofilm formation by three strains of *C. albicans* were investigated. Seven out of nine drugs tested at a concentration of 1 mM inhibited biofilm formation. Aspirin, etodolac and diclofenac produced the greatest effects, with aspirin causing up to 95% inhibition. Surviving cells had a wrinkled appearance, as judged by scanning electron microscopy, and consisted of both yeasts and hyphae.

The effect of aspirin on viability of both biofilm and planktonic cells was pH dependent, with the greatest effect at pH 3 or in unbuffered yeast nitrogen base medium. The effect of aspirin on metabolic activity and viability of mature *C. albicans* biofilms was similarly pH dependent. At pH 3, aspirin inhibited metabolic activity of mature *C. albicans* biofilms more than fluconazole or a mixture of fluconazole and aspirin. This study also demonstrated that both biofilms and planktonic cells of *C. albicans* synthesize extracellular prostaglandin(s) during growth at 37°C, but biofilm cells secrete significantly more when production is determined on the basis of cell dry weight. Prostaglandin synthesis by both cell types was sensitive to the cyclooxygenase inhibitors aspirin, diclofenac and etodolac. A morphological mutant blocked in two signalling pathways (*cph1/cph1 efg1/efg1*) produced prostaglandin levels similar to those of the parent strain but formed yeast-only biofilms.

Unicellular organisms such as fungi have been reported to interact with hormones, such as fungal sex hormones and mammalian hormones. In this study the effect of several steroids including progesterone, corticosterone, dexamethasone, prednisolone, hydrocortisone and estradiol on *C. albicans* biofilm development was investigated. The results showed that biofilm formation was not affected by any of these compounds. However, at 1μM some steroids such as progesterone and dexamethasone inhibited germ-tube formation by more than 25%. At 100 to 1000 μM, all steroids inhibited germ-tube formation.

INTRODUCTION

1. *Candida* and Candidosis

1.1 *Candida* species

Mycoses caused by opportunistic fungal pathogens have increased dramatically in recent years and the most regularly encountered species are members of the genus *Candida*. *C. albicans* is by far the most common *Candida* species causing infections in humans, followed by *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, and C. *guilliermondii* (Pfaller *et al.*, 1998; Pfaller *et al.*, 2001). *C. dubliniensis* is a new, recently described species, which was originally isolated from severe oral lesions in subjects infected with human immunodeficiency virus (HIV) and was therefore suspected of increased virulence (Moran *et al.*, 1997).

C. albicans is a fungus which may develop into a number of different morphological forms, including yeasts, hyphae and pseudohyphae. This change depends on environmental conditions. The ability to form hyphae is generally considered to be an important virulence factor since hyphae are able to penetrate tissue more easily. *Candida* infection is known as candidosis or candidiasis (Odds, 1988; Bodey, 1993).

1.2 *Candida* infections

Candida species are usually harmless members of the flora of the skin, mucous membranes, or gastrointestinal tract; under normal circumstances they do not produce clinical disease (Odds, 1988). However, they may become opportunistic pathogens under certain conditions. The increased incidence of *Candida* infection has been attributed to widespread use of antibiotics (Mitchell, 1998; Sanchez-Martinez and Perez-Martin, 2001; Epstein *et al.*, 2002) , the rise of

AIDS, a larger number of organ transplantations (Haynes, 2001), and the common use of invasive devices (catheters, artificial joints and valves), all of which increase a patient's susceptibility to infection. Candidosis in clinical disease includes two types of infection, superficial and systemic.

1.2.1 Superficial candidosis

Superficial candidosis includes cutaneous and mucosal *Candida* infections which are common and affect both immunocompromised and non-immunocompromised hosts. These infections involve the skin, nails, and oral and vaginal mucous membranes. For example, *Candida* paronychia is one of the most common infections of persons whose hands are frequently immersed in water. The most common oropharyngeal candidoses are oral thrush and denture-induced stomatitis. Oral thrush consists of white discrete plaques on an erythematous background on the buccal mucosa, throat, and tongue. This infection most commonly affects newborns, the elderly, and diabetics. In the absence of other known causes of immune suppression, oral thrush in an adult is highly suggestive of HIV infection (Dronda *et al.*, 1996).

Denture stomatitis, presenting as either a smooth or granular erythema confined to the denture bearing area of the hard palate is often associated with angular cheilitis. Symptoms vary, ranging from none to a sore and painful mouth, with a burning tongue and altered taste (Webb *et al.*, 1998). *C. albicans* has been described as the organism most often isolated from denture stomatitis (Sangeorzan *et al.*, 1994; Rioboo-Crespo Mdel *et al.*, 2005). However, other organisms such as *C. dubliniensis* and *C. glabrata* have emerged as causative agents among immunocompromised patients (Redding, 2001).

In woman, vaginal candidosis is another common infection; three quarters of all healthy women of child-bearing age experience at least one episode of vaginal candidosis, and approximately 5% of women with a primary episode subsequently develop distressing vaginal candidosis especially during pregnancy (Calderone, 2002). Although in the past *C. albicans* was recognized as the most frequent aetiological agent of vaginal infections, an increasing percentage of infections are now ascribed to non-*C. albicans* species, such as *C. tropicalis*, *C. glabrata* and *C. parapsilosis* (Karaca *et al.*, 2005), which are usually less sensitive to the most commonly used antifungal agents (White *et al.*, 2001).

1.2.2 Systemic candidosis

The second type of *Candida* infection is systemic candidosis. Systemic invasion by *C. albicans* is a very serious condition and occurs in patients with profound immunodeficiency. This immunodeficiency is usually secondary to AIDS, chemotherapy for malignant disease or terminal leukemia. These patients can have lesions in the lungs, liver, kidney, or be suffering from urinary tract infection, endocarditis, meningitis, or candidemia. The diagnosis is usually made by history, the demonstration of lesions and the appearance of circulating *Candida* antigens (Odds, 1988). Infections caused by non-*C. albicans* species have increased dramatically in the last five years due to inherent resistance to fluconazole by some of these species (Hobson, 2003). Therapy for serious *Candida* infections has been difficult because of the limited number of antifungal agents available. Although long the mainstay of treatment, amphotericin B is associated with toxicities (side effects) and requires intravenous administration. In addition, not long after the introduction of fluconazole, reports of clinical failure

in association with elevated MICs of fluconazole therapy began to appear. In most cases patients were suspected to be infected with an inherently azole-resistant species such as *C. glabrata* or *C. krusei* (Rex *et al.*, 1995). The frequency of various species of *Candida* causing bloodstream infections has been investigated over a three-year period (1997-1999). It was found that the rank order of the various species differed according to geographic location. For example, in the United States yeast bloodstream infections were caused primarily by *C. albicans*, followed by *C. glabrata*, *C. parapsilosis* and *C. tropicalis*, whereas in Latin America yeast bloodstream infections were caused by *C. albicans*, followed by *C. parapsilosis*, *C. glabrata* and *C. tropicalis* (Pfaller *et al.*, 2001).

1.2.3 Implant-associated *Candida* infections

The rapid development of modern medicine has led to the challenging problem of implant-associated infections. For example, catheter-related infections increased in incidence during the past decade, causing significant morbidity and mortality. Catheter insertions are often succeeded by bacterial or *Candida* adhesion and subsequent colonization of the catheter (Nicolle, 2005). A direct link has been demonstrated between nosocomial bloodstream infections and long-term indwelling intravascular catheter usage, especially in intensive care unit patients. Implant-associated infections often represent a diagnostic challenge due to the lack of consensus definition of what constitutes an infection, and in all cases the treatment of catheter-related sepsis is based on catheter removal (Worthington and Elliott, 2005).

The skin around the insertion site is the most common portal of entry. For example, in the case of vascular catheters, skin organisms migrate from the skin

insertion site along the external surface of the catheter and colonize the distal tip causing a bloodstream infection. The mortality rate of *Candida* catheter-associated infection is high and these organisms now rank as the fourth most common cause of bloodstream infection (Kojic and Darouiche, 2004). Unlike intravascular and urinary catheters, other devices such as hip and knee joint prostheses are not considered to be so liable to *Candida* infection (Kojic and Darouiche, 2004).

Several studies have led to proposals for improving the performance of catheters by reducing the microbial colonization of catheter surfaces. These include hydrophilic coatings (Schierholz *et al.*, 1999a; Schierholz *et al.*, 1999b) or using catheters containing antimicrobial agents. Future preventative strategies may also include the application of low voltage electric current in combination with antimicrobials (Elliott and Tebbs, 1998).

1.3 Predisposing factors for *Candida* infection

Pathogenesis involves a complex interrelationship between *Candida* species and the host (van Burik and Magee, 2001). There is no single factor that causes or permits *C. albicans* to be an agent of diseases that range from superficial to invasive human infection (Latge and Calderone, 2002). However, it is indigenous to humans and a part of the normal flora, and under conditions favouring its proliferation it can strike as a formidable pathogen.

There are numerous predisposing factors that lead *Candida* infections. First, disturbance of the epithelial barrier may occur as a result of indwelling catheters, peritoneal dialysis, burns, ulcers, trauma, or surgery (Kontoyiannis *et al.*, 2001). Second, defects or dysfunction of mononuclear phagocytes plus other cells may

be caused by chemotherapy, radiotherapy, aplastic anaemia, chronic granulomatous disease and diabetes mellitus (Singh, 2001; Bodey *et al.*, 2002). Third, defects or dysfunction of T-lymphocyte cell-mediated immunity may be caused by Hodgkins disease, transplantation, leukaemia and corticosteroids (Gosden *et al.*, 1997). Finally, suppression of the normal human flora may result from treatment with broad spectrum antibiotics allowing *Candida* to proliferate (Charles *et al.*, 2005).

1.4 Treatment and antifungal agents

Treatment of superficial and systemic *Candida* infections is limited to four classes of antifungal agents; these are polyenes, fluorinated pyrimidines, azoles and the recent class of echinocandins (Fromtling, 1988; Slavin *et al.*, 2004). Polyene drugs are fungicidal and have the broadest spectrum of activity of any clinically useful antifungal agent. Amphotericin B is the only drug of this type used for systemic infections, but it has both acute and chronic side effects such as nephrotoxicity (Odds, 1988). This agent binds sterol components in the cell membrane and alters the membrane permeability, allowing leakage of cytoplasmic components (Rex *et al.*, 1993; Schmitt, 1993). Although amphotericin B has been used for several decades, new developments have been undertaken to reduce its nephrotoxicity which can lead to acute renal failure. As a result, three lipid formulations of amphotericin B are now licensed, all of which show substantially less nephrotoxicity than the classical amphotericin B (Wingard and Leather, 2004).

5-Fluorocytosine (5FC) is the only antifungal compound available in the fluorinated pyrimidine class and is fungicidal, with a limited activity spectrum.

This drug interferes with pyrimidine metabolism of RNA at one or multiple enzymatic sites. It is most often used clinically in combination with amphotericin B because of the rapid development of resistance.

Azoles are the third class of antifungal agents and all members of this class are fungistatic (Warnock, 1992). They include ketoconazole, miconazole, clotrimazole, itraconazole and fluconazole. Azole antifungal agents prevent the synthesis of ergosterol (Warnock, 1992), a major component of fungal plasma membranes, by inhibiting the cytochrome P-450-dependent enzyme, lanosterol demethylase (Fromtling, 1988). This enzyme also plays an important role in cholesterol synthesis in all eukaryotic cells (Odds, 1988). In the past few years, new azoles have been produced, including voriconazole which shows a wider spectrum of activity against yeasts and filamentous fungi (Canuto and Rodero, 2002).

Caspofungin is the first promising member of the echinocandins, the newest class of antifungal agents. It inhibits the synthesis of β-1,3-D-glucan in the cell wall of numerous pathogenic fungi such as *Candida* and *Aspergillus* species. This represents a novel and unique target which is not found in mammalian cells. Therefore caspofungin is capable of selective toxicity. For example, recent *in vitro* studies suggested that caspofungin is less nephrotoxic than amphotericin B (Wegner *et al.*, 2005).

Despite recent progress, there are still relatively few antifungal drugs, especially when compared with the number of antibacterial drugs (Fromtling, 1988). Drug targets that distinguish pathogen from host are more difficult to identify in fungi than in bacteria, at least in part because fungal cells, like animal

cells, are eukaryotic, whereas bacteria are much more distantly related to their human hosts (Bodey, 1993). Because many potential antifungal drug targets have homologues of similar function and susceptibility to inhibition in humans, toxic side effects dramatically reduce the number of antifungal agents that can be used therapeutically (Graybill, 1992).

2. Virulence factors of *Candida* species

2.1 Adhesion

Adhesion of *C. albicans* to host cells is believed to represent a fungal virulence factor and a significant step in the development of candidosis (Odds, 1988; Bodey, 1993). Therefore there is a strong correlation between virulence and adhesion of *C. albicans* to host cells. Moreover, adhesive properties of the different *Candida* species match their virulence ranking. Adhesion to epithelial and endothelial cells, fibrin-platelet matrices, bacteria, neutrophils and vaginal mucosae has been extensively studied and a close correlation was observed between the adhesion of various *Candida* species and their ability to cause disease (Robert *et al.*, 2000).

C. albicans can also bind to several extracellular matrix proteins of mammalian cells, such as fibronectin, laminin and fibrinogen (Fromtling, 1988). Many studies have shown that several factors influence adhesion *in vitro*. For example, the composition of the growth medium can affect adhesion; yeasts grown in medium containing 500 mM galactose or glucose were significantly more adherent than organisms grown in a low concentration (50 mM) of glucose (McCourtie and Douglas, 1984; Pizzo *et al.*, 2000). Adherence to exfoliated

epithelial cells can vary significantly between epithelial-cell donors, from day to day with the same donor, and with changes in the menstrual cycle (Theaker *et al.*, 1993). Pre-treatment of *C. albicans* cells with whole saliva promotes adherence to cultured epithelial cells (Holmes *et al.*, 2002). Sub-inhibitory concentrations of antifungal agents such as amphotericin B inhibit *C. albicans* adherence to polystyrene and fibrinogen surfaces, while improving adherence to surfaces coated with extracellular matrix gel (Imbert *et al.*, 2002).

2.1.1 Adhesins and host cell receptors

The cell surface of *C. albicans* is the key factor for the contact between this yeast and host defences. The host response to *Candida* infection is the result of a complex interaction between the pathogen and the host's innate and adaptive immune system. Cell-mediated immunity is widely considered to be critical for the successful outcome of fungal infections. Physical interactions of this fungus with the host are mediated at the cell surface, and cell wall constituents implicated in binding have been designated adhesins (Masuoka, 2004). There are several types of host – pathogen interactions; these include protein-protein, protein-carbohydrate and carbohydrate-carbohydrate interactions (Pendrak and Klotz, 1995).

The major components of the cell wall are glucan, chitin, and mannoproteins. The outer, surface mannoproteins have been implicated in adhesion to host tissues and ligands. Cell surface mannoproteins contain both *O*- and *N*-linked oligosaccharides (Calderone, 2002). Recent evidence has shown that both oligosaccharides can serve as adhesins in the attachment of *C. albicans* yeast cells to the host. *C. albicans* mutants deleted in *MNT1* and/or *MNT2* genes which

are involved in the formation of *O*-linked oligosaccharides showed significantly reduced adhesion to human buccal epithelial cells and were less virulent (Buurman *et al.*, 1998; Munro *et al.*, 2005). A *C. albicans* null mutant lacking the *OCH1* gene had a clear defect in the *N*-glycan outer chain and correspondingly displayed a weakened cell wall. The *N*-glycan outer chain was shown to be necessary for normal host-fungal interactions and virulence (Bates *et al.*, 2006).

In early studies, exfoliated buccal epithelial cells were the most widely used human cell types in *C. albicans* adherence experiments. Consequently, many receptors for *Candida* on these buccal cells have been proposed. For example, fibronectin was the first molecule to be recognized as a receptor for *C. albicans* (Skerl *et al.*, 1984), The role of glycosphingolipids as adhesion receptors for *C. albicans* was examined; Jimenez-Lucho *et al.*(1990) showed yeast cells of *C. albicans* bound specifically to lactosylceramide. Lactosylceramide is a major glycosphingolipid in these epithelial cells and the only one to which the yeasts bound. As lactosylceramide is widely distributed in epithelial tissues, the authors suggested that this glycosphingolipid may be the receptor for yeast colonization and disseminated disease in humans (Jimenez-Lucho *et al.*, 1990).

Interaction with components of the complement system is an important aspect of the pathogenesis of infection by *C. albicans*. Pseudohyphal but not yeast forms of *C. albicans* possess both iC3b and C3d receptors indicating that the expression of the functionally active iC3b receptor on *C. albicans* may be involved in the virulence of the organism, possibly by mediating adherence to mammalian cells such as neutrophils cells (Alaei *et al.*, 1993). Moreover, candidal lectin-like epithelial adhesins that recognize L-fucose or GlcNAc have been

reported (Critchley and Douglas, 1987a; Critchley and Douglas, 1987b). The binding of some *C. albicans* strains to buccal epithelial cells was inhibited by fucose, but in other strains it was inhibited by glucosamine or GlcNAc, suggesting strain-specific receptors.

Synthesis of the lectin-like material increased when organisms were grown on galactose (McCourtie and Douglas, 1985). Fucose has been shown to bind to *C. albicans* germ-tube tips (Vardar-Unlu *et al.*, 1998). An L-fucose-binding protein was purified by affinity chromatography with the blood group H trisaccharide antigen which suggested that blood group antigens may act as epithelial cell receptors for *C. albicans* (Cameron and Douglas, 1996).

2.2 Dimorphism

The ability to switch between a yeast-like form and a filamentous form is a well known characteristic of *C. albicans*. (Odds, 1988; Mitchell, 1998; Liu, 2001). In pathogenic fungi, this capacity has been correlated with virulence because during the infection process, dimorphic transitions are often required. For example *C. albicans* mutant strains defective in germ-tube formation show decreased tissue invasion in an immunosuppressed animal model (Riggle *et al.*, 1999). Morphogenetic transformation of *C. albicans* is triggered by serum components, N-acetyglucosamine, and an elevated temperature. The organism switches from a unicellular yeast form to a multicellular hyphal form that supports the penetration of host cells or tissues (Ramon *et al.*, 1999).

In the host, hyphae may be suited to breach barriers, whereas the yeast form is more easily disseminated. Both yeast cells and filaments are found at infection sites, so it is reasonable to assume that both contribute to pathogenesis. The

hyphal form is important for penetrating tissue surfaces and escaping from host cells following internalization; these mechanisms enable hyphal invasion of epithelial and endothelial surfaces (Kumamoto and Vinces, 2005). Therefore, understanding the mechanisms for this morphogenetic switch should provide an insight into the pathogenicity of this fungus (Odds, 1988). During hyphal growth in *C. albicans*, cell surface expansion is restricted to a small region at the hyphal tip. This apical growth zone is active during the entire hyphal growth period (Liu, 2001). Increasing evidence supports the view that hyphal growth is a response to nutrient deprivation, and that filamentous growth enables the fungus to forage for nutrients more effectively (Ramon *et al.*, 1999).

2.3 Production of extracellular enzymes

The ability of *C. albicans* to produce extracellular hydrolytic enzymes such as secreted aspartyl proteinases (SAP), phospholipases and lipases, is an important factor which contributes to *C. albicans* virulence. For example, *C. albicans* isolates from patients with vaginal or oral candidosis produced more proteinases than *C. albicans* isolates from asymptomatic carriers (Naglik *et al.*, 2003). The role of secreted aspartyl proteinases enzymes in *C. albicans* infection is linked directly to virulence functions including hydrolysing proteins of host cell membranes, adhesion, and tissue invasion (Calderone, 2002). Secreted aspartyl proteinases are usually active at acidic pH. However, it has been demonstrated that aspartyl proteinases of *C. albicans* have at least 10 members (Sap 1-10). Sap 2 and Sap 4-6 have been reported to be more active at neutral pH (Wagner *et al.*, 1995), whereas Sap1–3 have their highest activity at lower pH values. These different pH optima of the Sap isoenzymes provide *C. albicans* with a range of proteolytic activity from pH 2.0 to 7.0. Different pH values can optimize different

proteinase enzymes which may explain the ability of *C. albicans* to infect a variety of tissues, as in vaginal (acidic pH) or oral candidosis (neutral pH) (Schaller *et al.*, 2005).

Four types of phospholipases have been reported in *C. albicans* - phospholipases A, B, C, and D - but only the *PLB1* and *PLB2* gene products have been detected extracellularly. Although *PLB1* is thought to account for most of the secreted phospholipase B activity in *C albicans*, *PLB2* contributes in a minor way because a *PLB1*-deficient strain still produces residual amounts of phospholipase B activity. Of the four types of phospholipase identified, PLB 1 and PLD1 have been shown to be required for virulence. In *C. albicans* the expression of this type of hydrolytic enzyme can be affected by several environmental factors, such as temperature, pH, and growth phase of *C. albicans*. Furthermore, it was found that yeast cells or hyphae expressed higher levels of PLB1 mRNA than germ-tube forming cells (Hoover *et al.*, 1998). Non-*C. albicans* species such as *C. tropicalis*, *C. glabrata* and *C. parapsilosis* have been reported to produce phospholipases (Fu *et al.*, 1997). Although deletion of *PLB1* did not produce any detectable effects on candidal adherence to human endothelial or epithelial cells, the ability of the *C. albicans* mutants lacking *PLB1* to penetrate host cells was dramatically reduced. Therefore, phospholipase B may well contribute to the pathogenicity of *C. albicans* by abetting the fungus in damaging and traversing host cell membranes, processes which likely increase the rapidity of disseminated infection (Leidich *et al.*, 1998).

In comparison to proteinases and phospholipases, little was known about *Candida* lipases or esterases until recently. Now, however, a range of lipase genes

(*LIP*1–10) has been identified in *C. albicans* (Hube *et al.*, 2000; Schofield *et al.*, 2005), as well as in other *Candida* species such as *C. parapsilosis* (Neugnot *et al.*, 2002; Stehr *et al.*, 2003). This enzyme enabled *C. albicans* to grow on lipids as the sole source of carbon (Roustan *et al.*, 2005). Moreover, secreted lipolytic enzymes of *C. albicans* have been discussed as virulence factors. Lipase genes constitute a large gene family that may have evolved to adapt to the permanent association of *C. albicans* with the human or animal host and may also have important functions during persistence and infection processes. It was found that lipase gene expression depended on the stage of infection rather than on the organ localization (Hube *et al.*, 2000).

2.4 *C. albicans* transcription factors

Inside the host, *Candida* species have to interact with different microenvironments characterized by many conditions such as nutrient availability, pH, and temperature (Calderone, 2002). Therefore *Candida* has to respond to these new situations by means of signal transduction pathways such as the mitogen-activated protein kinase (MAP kinase) pathway. The enzymes in these pathways are involved in regulating growth and differentiation processes by sensing environmental changes and transducing the signals detected at the cell surface to the interior of the cell by sequential protein activation (Brown and Gow, 1999). Since these signalling pathways were studied earlier in *Saccharomyces cerevisiae*, most investigations of mitogen-activated protein kinase (MAP kinase) signalling in *C. albicans* have used *S. cerevisiae* as a comparative model organism.

In *C. albicans*, morphological switching from yeasts to hyphae is dependent on stimulation of regulatory pathways. Several signalling pathways that regulate morphogenesis have been identified, including various transcription factors that either activate or repress hypha-specific genes. Two well-characterized pathways include the MAP kinase cascade and cAMP-dependent protein kinase pathway that regulate the transcription factors Cph1 and Efg1, respectively (Stoldt *et al.*, 1997; Banuett, 1998). The *cph1/cph1* mutant of *C. albicans* shows delayed initiation of filamentous growth on Spider agar plates, relative to wild-type cells, but shows wild-type levels of filamentous growth in response to liquid serum treatment (Liu *et al.*, 1994). In contrast, *efg1/efg1* mutant cells are strongly attenuated in responding to serum, showing no germ tubes or hyphae within 2 h of being introduced to 20% serum at 37°C. Additionally it was found that *efg1/efg1* mutant cells were reduced in virulence when evaluated in a mouse model experiment, as was the double mutant strain *efg1/efg1 cph1/cph1*. This observation suggested that *EFG1* and *CPH1* together account for the pathways that activate filamentous growth in *C. albicans* (Lo *et al.*, 1997; Stoldt *et al.*, 1997).

3. Microbial biofilms

Biofilms are microbial communities characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan, 2002; Hall-Stoodley and Stoodley, 2002). It is clear that microorganisms undergo profound changes during their transition from planktonic (free-

swimming) organisms to cells that are part of a complex, surface-attached community (Costerton, 1995). Biofilms can be defined simply and broadly as communities of microorganisms that are attached to a surface.

Using tools such as the scanning electron microscope and, more recently, the confocal laser scanning microscope (Moller *et al.*, 1996), biofilm researchers now understand that biofilms are not unstructured, homogeneous deposits of cells and are phenotypically different from planktonic cells (Kolenbrander, 1997; Baillie and Douglas, 1998a; Okabe *et al.*, 2001; Donlan and Costerton, 2002). Bacterial biofilms are the most commonly studied because of their role in colonizing medical implants such as prosthetic heart valves and joint replacements. Biofilms can comprise a single microbial species or multiple microbial species and can form on a range of biotic and abiotic surfaces (Donlan and Costerton, 2002). However, when they are nutrient deprived, biofilm cells can detach from the surface and return to a planktonic mode of growth; this behaviour may be the first step towards colonization of another surface (Stickler *et al.*, 1998; Hunt *et al.*, 2003). Recent reports suggest different strategies by which cells from bacterial biofilms disperse or detach; for example, in *Pseudomonas aeruginosa* biofilm cells disperse by twitching motility (swarming), but in *S. aureus* biofilms cells disperse by rolling motility (clumping) (Hall-Stoodley and Stoodley, 2005).

Adherence is the first stage in the development of a biofilm on biomaterials in device-related infections. It may be influenced by many factors such as biomaterial surface characteristics, growth conditions and biomaterial surface chemistry. Donelli and Francolini (2001) have reported that intravascular catheter–related infections are a major cause of morbidity and mortality in the

United States. More than 200,000 nosocomial bloodstream infections occur each year in the United States; most of these infections are related to different types of intravascular devices - in particular, the nontunnelled central venous catheter (Donelli and Francolini, 2001). The risk factors for intravenous catheter–related infections vary according to the type of catheter, the hospital size or unit, the location of the site of catheter insertion, and the duration of catheter placement.

4. *Candida* biofilms

The first report of biofilm formation by *Candida* species *in vitro* was by Hawser and Douglas (1994). A model system was devised for studying *Candida* biofilms growing on the surface of small disks of catheter material. Since that time, a variety of model systems have been developed.

4.1 Biofilm model systems

Most methods for examining growth of *Candida* biofilms have been modified from those used in early studies on bacterial biofilms. The catheter disk model was the first to be used; adhered cells of *Candida* were allowed to grow on the surface of polyvinylchloride (PVC) catheter disks and biofilm activity was monitored quantitatively by MTT or XTT tetrazolium salt reduction which is a colorimetric method, or by measuring biofilm cell dry weight (Hawser and Douglas, 1994). In other studies, a denture acrylic strip model was established to investigate *C. albicans* biofilms (Nikawa *et al.*, 1996; Chandra *et al.*, 2001b), while microtitre plates (96-well) were used to examine *Candida* biofilms in the presence of antifungal agents because large numbers of biofilm samples can be processed in this way (Ramage *et al.*, 2001b). *Candida* biofilms have also been

evaluated under liquid flow conditions which relate to many biofilms formed *in vivo*; for example, the perfused biofilm fermenter (Baillie and Douglas, 1998a) , the cylindrical cellulose filter (Baillie and Douglas, 1998b) and more recently the CDC biofilm reactor system (Honraet *et al.*, 2005) are systems used for developing biofilms under flow conditions. Recently, *C. albicans* biofilms were investigated *in vivo* by using a rat central venous catheter model (Andes *et al.*, 2004) and a rabbit central venous catheter model (Schinabeck *et al.*, 2004).

4.2 Factors affecting the ability of *Candida* to form biofilms

Several *in vitro* studies have demonstrated a variety of factors affecting biofilm formation. Using the catheter disk model, Hawser and Douglas (1994) showed that there was some correlation between the ability to form biofilms and pathogenicity when different *Candida* species were investigated. For example, isolates of *C. parapsilosis*, *C. pseudotropicalis* and *C. glabrata* all gave significantly less biofilm growth than the more pathogenic *C. albicans*. This observation was confirmed by another report (Kuhn *et al.*, 2002a) which showed that *C. albicans* produces quantitatively larger and qualitatively more complex biofilms than other species, in particular, *C. parapsilosis*. Moreover, minimal variations in biofilm production among invasive *C. albicans* isolates were observed, but significant differences between invasive and noninvasive isolates were seen (Kuhn *et al.*, 2002a). Recently, it has been shown that there is a correlation between biofilm production by different *C. parapsilosis* strains and strain genotype. A majority of *C. parapsilosis* isolates from the bloodstream, hands of health-care workers, and all other sites from patients belonged to one genotype group and had the ability to produce biofilms, while other strains had different genotypes and failed to produce biofilms (Song *et al.*, 2005).

The nature of the catheter material used *in vitro* can also affect biofilm formation by *C. albicans*. For instance, evaluation of various catheter materials showed that biofilm formation by *C. albicans* was slightly increased on latex or silicone elastomer, compared with polyvinyl chloride, but substantially decreased on polyurethane or 100% silicone (Hawser and Douglas, 1994). *In vivo*, implants rapidly adsorb host proteins, which form a conditioning film on the surface. Nikawa *et al.* (1996) examined the effect of modification of denture acrylic surfaces on biofilm formation by *C. albicans*; they found that biofilm formation was promoted when the surfaces were pre-coated with saliva or serum.

4.3 Biofilm ultrastructure

After 48h, *C. albicans* biofilms on catheter disks consist of a dense network of yeasts, germ tubes, pseudohyphae, and hyphae as examined by scanning electron microscopy (Hawser and Douglas, 1994). A subsequent study demonstrated that *C. albicans* biofilms produce more extracellular matrix material when incubated with gentle shaking instead of statically (Hawser *et al.*, 1998). Biofilms of *C. albicans* on catheter disks produce two layers: a thin, basal yeast layer and a thicker, hyphal layer (Fig. 1). The basal yeast layer is thought to play an important role in anchoring the biofilm of *C. albicans* to the surface (Baillie and Douglas, 1999b; Douglas, 2003). Subsequent studies confirmed that *in vivo* *C. albicans* biofilm structure was similar to biofilm architecture for *in vitro* models, but that in addition, host cells were embedded in the extracellular matrix (Andes *et al.*, 2004).

Using fluorescence and confocal scanning laser microscopy, Chandra and *et al* (2001) demonstrated that biofilm formation proceeds through three distinct

developmental phases. These growth phases transform adherent blastospores to well-defined cellular communities encased in a polysaccharide matrix (Chandra *et al.*, 2001a). However, *C. albicans* biofilms have a morphology different from that of other species, consisting of a basal blastospore layer with a dense overlying matrix composed of exopolysaccharides and hyphae. In contrast, *C. parapsilosis* biofilms are composed exclusively of clumped blastospores (Kuhn *et al.*, 2002a).

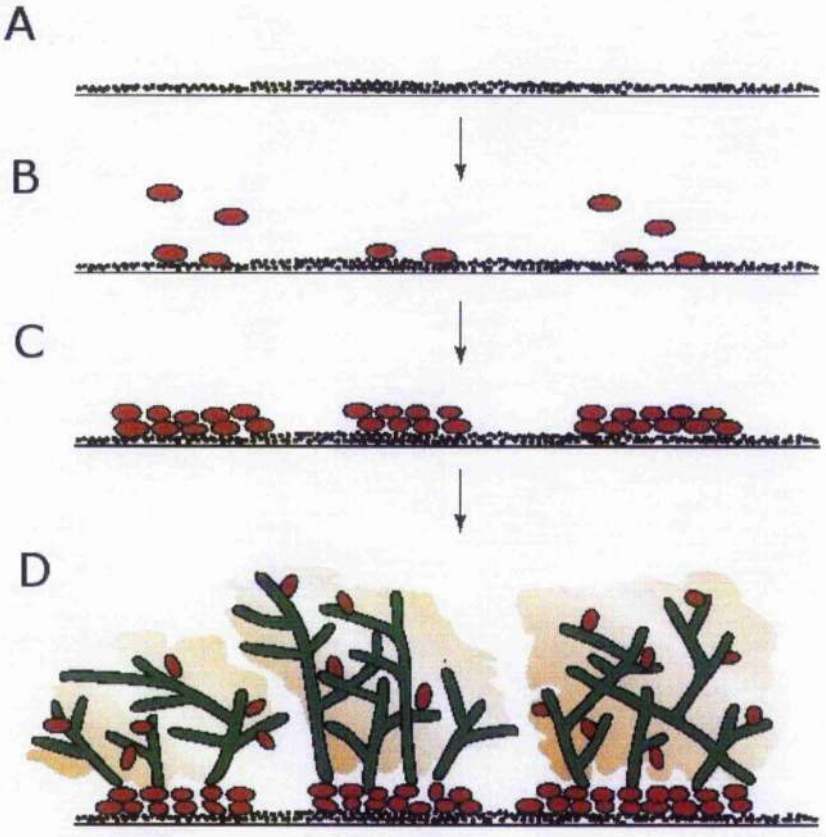
Unlike the scanning electron microscope, confocal scanning laser microscopy used with fluorescent dyes allows the examination of living biofilms in a fully hydrated condition. When examined by this technique, *C. albicans* biofilms displayed complex three-dimensional structures consisting of microcolonies of yeasts and hyphae embedded in matrix material, interlaced with water channels which maintain a mechanism for nutrient circulation inside the biofilm (Ramage *et al.*, 2001c; Fig 1).

Recent investigations at the level of morphogenetic signalling pathways have provided evidence of a role for these pathways in controlling the structure of *C. albicans* biofilms. Mutant strains of *C. albicans* lacking the *EFG1* gene were incapable of producing hyphae or forming biofilms (Ramage *et al.*, 2002b). In another study, mutants lacking the transcription factors Efg1 and Cph1 colonized polyurethane catheters very poorly compared to the wild type strain (Lewis *et al.*, 2002). On the other hand, in a recent report it was shown that *efg1/cph1* mutants produced yeast-only biofilms on a glass surface (Garcia-Sanchez *et al.*, 2004).

Figure 1

Stages in the formation of a *C. albicans* biofilm

Stages in the formation of a *C. albicans* biofilm on a polyvinylchloride (PVC) catheter surface. (A) Catheter surface with an adsorbed conditioning film of host proteins (black dots). (B) Initial yeast (red) adhesion to the surface. (C) Formation of the basal layers of yeast microcolonies. These anchor each microcolony to the surface. (D) Completion of microcolony formation by addition of the upper, mainly hyphal layer and matrix material (yellow) that surrounds both yeasts (red) and hyphae (green). Mature biofilms contain numerous microcolonies with interspersed water channels to allow circulation of nutrients. On other surfaces (e.g. cellulose fibres) microcolonies consisting entirely of yeast cells are produced (Douglas, 2003).


A
B
C
D

4.3.1 Mixed fungal-bacterial biofilms

Although most studies have investigated the structure of *Candida* biofilms formed by a single species, mixed species fungal-bacterial biofilms have been reported. For example, in an *in vitro* model of mixed species biofilms, two different strains of *Staphylococcus epidermidis*, a slime-producing wild type and a slime-negative mutant, showed extensive interactions with *C. albicans*. These interactions were more easily seen with the slime-negative mutant. Staphylococci were present beneath and above the yeast and hyphal layers, and were clearly adherent to both morphological forms of the fungus. All three species when grown individually showed maximum biofilm growth up to 24 h, but mixed species biofilms continued to grow up to 48 h (Adam *et al.*, 2002).

Another report investigated the interactions between *C. albicans* and 12 other species of *Candida* and bacteria in biofilms. The number of cells within biofilms grown in a polystyrene tube model was measured after adding *C. albicans* to preformed biofilms of the other microorganisms, and vice versa. The number of cells of *C. albicans* within the growing biofilms decreased significantly when the fungus was added to preformed biofilms of *Candida* species and bacteria except with *C. parapsilosis, C. glabrata* or *P. aeruginosa* (a slime producer). However, when *C. parapsilosis*, *S. epidermidis* (a slime-negative strain) or *Serratia marcescens* was added to preformed biofilms of *C. albicans*, the number of cells of these microorganisms increased in the growing biofilms (El-Azizi *et al.*, 2004). The authors concluded that biofilms of *C. albicans* are capable of holding other microorganisms and more likely to be heterogeneous with other bacteria and fungi in the environment and on medical devices.

4.4 Drug resistance of biofilms

The drug resistance of *Candida* biofilms has been demonstrated by several studies. Hawser and Douglas (1995) showed that biofilms formed by *C. albicans* and other *Candida* species were resistant to the action of five clinically important antifungal agents, including amphotericin B and fluconazole, when compared with planktonic cells. Drug concentrations required to reduce metabolic activity by 50% were five to eight times higher for biofilms than for planktonic cells, and 30–2000 times higher than the corresponding minimum inhibitory concentrations (MICs) (Hawser and Douglas, 1995). A subsequent study used a cylindrical cellulose filter method to investigate biofilm formation by *C. albicans* at low growth rate under iron- and glucose- limitation. It was found that both biofilm types were resistant to amphotericin B, but daughter cells from iron-limited biofilms were significantly more susceptible to the drug than those from glucose-limited biofilms (Baillie and Douglas, 1998b). A microtitre-based colorimetric assay has also been used for susceptibility testing of *Candida* biofilms. Ramage *et al.* (2001b) confirmed that biofilms from different *C. albicans* strains were resistant to amphotericin B and fluconazole. Amphotericin B was up to 32 times less active against biofilms than against planktonic cells. However, amphotericin B still demonstrated some activity against *C. albicans* biofilms, as indicated by the biofilm MIC_{50} values (Ramage *et al.*, 2001b).

Subsequent studies showed that biofilms formed by *C. albicans* on denture acrylic are highly resistant to antifungal agents such as fluconazole, nystatin, amphotericin B and chlorhexidine (Chandra *et al.*, 2001b). Similarly, biofilms formed by *C. albicans* and *C. parapsilosis* on silicone were reported to be resistant two new triazoles (voriconazole and ravuconazole). In contrast, lipid

formulations of amphotericin B (liposomal amphotericin B and amphotericin B lipid complex), and echinocandins such as caspofungin and micafungin, showed activity against *Candida* biofilms (Bachmann *et al.*, 2002; Kuhn *et al.*, 2002b).

4.5 Possible mechanisms of biofilm drug resistance

The mechanisms by which *Candida* biofilms are resistant to antifungal agents remain to be elucidated. Possible mechanisms include: restricted drug penetration through biofilm matrix material; a slow growth rate which can produce phenotypic changes; and differential gene expression when biofilms interact with different surfaces (Mah and O'Toole, 2001). The presence of "persister" cells has also been suggested recently (Lewis, 2005).

4.5.1 Restricted drug penetration through biofilm matrix material

Matrix material, which is one of the distinguishing characteristics of biofilms, has long been held to have a role in preventing the diffusion of antibiotics into microbial biofilms and acting as a diffusion barrier (Stewart, 1996; Gilbert *et al.*, 2002). To determine whether the matrix material is involved in the resistance of *Candida* biofilms, Baillie and Douglas (2000) compared the susceptibility of *Candida* biofilms grown statically (which possessed a sparse matrix) with those of biofilms incubated with gentle shaking (which produced copious amounts of matrix material). The extent of matrix formation did not significantly affect the susceptibility of *C. albicans* biofilms to amphotericin B , flucytosine or fluconazole (Baillie and Douglas, 2000). However, other investigations with biofilms produced under flow conditions suggested that matrix material may have a minor role in biofilm resistance, since resuspended cells (which had lost most of their matrix material) were 20% less resistant to

amphotericin B than intact biofilms (Baillie and Douglas, 1998a; Baillie and Douglas, 1998b).

Other studies have been carried out in which antifungal penetration has been assessed by detecting the concentration of the drug able to penetrate the biofilm. Al-Fattani and Douglas (2004) used a filter disk assay to investigate the penetration of flucytosine, fluconazole, amphotericin B, and voriconazole through single- and mixed-species biofilms containing *Candida*. They found that fluconazole permeated all single-species *Candida* biofilms more rapidly than flucytosine. Either drug showed similar diffusion rates through biofilms of three strains of *C. albicans*. However, the rates of drug diffusion through biofilms of *C. glabrata* or *C. krusei* were faster than those through biofilms of *C. parapsilosis* or *C. tropicalis*. In all cases, after 3 to 6 h the drug concentration at the distal edge of the biofilm was very high but failed to produce complete killing of biofilm cells (Al-Fattani and Douglas, 2004). Related observations were subsequently reported with a similar model system; fluconazole and flucytosine demonstrated similar levels of perfusion, while amphotericin B showed the lowest penetration through biofilms of *C. albicans*, *C. parapsilosis*, and *C. krusei* (Samaranayake *et al.*, 2005). However, both investigations indicated that poor antifungal penetration is not a major drug resistance mechanism for *Candida* biofilms.

4.5.2 Slow growth rate

Slow or no growth is generally accompanied by an increase in the resistance of microorganisms to antibiotics. Cells growing in biofilms are usually exposed to some form of nutrient limitation. Therefore it has been suggested that physiological changes resulting from a slow growth rate can account for the

resistance of biofilms to antimicrobial agents (Mah and O'Toole, 2001). This possibility has been investigated in *Candida* biofilms; a perfused biofilm fermenter was used by Baillie and Douglas (1998a) to assess whether the growth rate of *C. albicans* biofilms could affect their susceptibility to antifungal agents. The method used allowed growth-rate control of adherent microbial populations. It was found that biofilms formed at different growth rates were resistant to amphotericin B, whereas planktonic cells were resistant only at very low growth rates (Baillie and Douglas, 1998a). Subsequent studies showed that *C. albicans* biofilms grown on cylindrical cellulose filters under iron- and glucose-limitation were equally resistant to amphotericin B. However, daughter cells from iron-limited biofilms were significantly more susceptible to the drug than those from glucose-limited biofilms (Baillie and Douglas, 1998b).

4.5.3 Differential gene expression

The multidrug resistance phenotype in planktonic cells of *C. albicans* has previously been shown to be due to overexpression of several genes known to be involved in azole resistance, including *CDR1*, *CDR2* and *MDR1*. These genes are upregulated leading to the activation of plasma membrane efflux systems which pump the drugs from the cells. Overexpression of drug efflux pumps has been demonstrated in *C. albicans* biofilms. However, strains carrying single or double mutations in these genes retained a resistant phenotype during biofilm growth (Ramage *et al.*, 2002c). Subsequent studies by Mukherjee *et al.* (2003) showed that biofilms formed by *C. albicans Cdr1 /Cdr2* and *Cdr1 /Cdr2/ Mdr1* double and triple mutants, respectively, were more susceptible to fluconazole than the wild-type strain, but at later time points (12 and 48 h), all the strains became

resistant to fluconazole. These observations indicate a lack of involvement of efflux pumps in resistance at late stages of biofilm formation, while demonstrating that additional, as yet unidentified, resistance mechanisms are involved in *Candida* biofilm formation.

Recently, differential gene expression was investigated in planktonic cells of *C. albicans* and biofilm-associated cells grown *in vivo* in a rat model system. Using quantitative RT-PCR it was found that there was no difference in expression of the azole target enzyme gene *ERG11*, or *MDR1*, between planktonic cells and biofilm-associated cells. However, mRNAs from both of the ATP-binding cassette pumps, *CDR1* and *CDR2*, were significantly increased in the biofilm state. *CDR2* was the most remarkably affected, with a nearly 10-fold increase in expression (Andes *et al.*, 2004).

4.5.4 "Persister" cells

"Persister" is the term used for subpopulations of microorganisms known for high tolerance to killing by antibiotics or that neither grow nor die in the presence of antibiotics. Lewis (2001) suggested that that there are phenotypic changes in microbial biofilms which result in the expression of biofilm-specific resistance genes or the presence of persister cells. The dynamics of persister formation are clearly more complex in biofilms. It has been suggested that persisters can accumulate within the biofilm, although the production of persisters near the surface of the biofilm is outpaced by reproduction of susceptible bacteria. Therefore persister cells may be contributing factors in the time-dependent tolerance of both planktonic cells and biofilms to high concentrations antimicrobial agents (Harrison *et al.*, 2005; Roberts and Stewart, 2005).

Recently, it has been suggested that biofilms can produce more persister cells than can planktonic organisms (Lewis, 2005). In addition, it has been shown that the fraction of persister cells in bacterial biofilms increased during incubation and that more persister cells were observed in thicker biofilms (Roberts and Stewart, 2005). Since the hypothesis of persister cells in bacterial biofilms has not been thoroughly investigated, it is not known whether the same hypothesis can be applied to *Candida* biofilms.

5. Quorum sensing

Quorum sensing was first described in 1970 by Nealson *et al.* in planktonic cells of the luminescent marine bacterium *Vibrio fischeri*. This bacterium produces a diffusible compound, termed an autoinducer, that accumulates in the surrounding environment during growth. At low cell densities the autoinducer is at low concentrations, and at high cell densities the autoinducer can accumulate to a concentration sufficient for activation of the *lux* genes (responsible for light production). At that time this report led to the theory of cooperative behaviour between cells of *V. fischeri* in response to the accumulation of secreted autoinducer signalling molecules (Nealson and Hastings, 1979). It is now clear that some of the functions of biofilms depend on the ability of the bacteria and microcolonies within the biofilm to communicate with one another. Quorum sensing is dependent on cell density and involves the regulation of expression of specific genes through the accumulation of signalling compounds that mediate intercellular communication (Miller and Bassler, 2001). With few cells, signalling compounds may be produced at low levels; however, autoinduction leads to increased concentration as cell density increases. Once the signalling compounds

reach a threshold level (quorum cell density), gene expression is activated (Finch *et al.*, 1998).

Cell signalling has been studied extensively in luminescent bacteria and appears to be mediated by an acyl homoserine lactone (AHL) encoded by the *lux* I gene. A similar system has been shown to exist in certain other Gram-negative species (Nilsson *et al.*, 2001). The high cell concentrations in biofilms present an ideal situation for quorum sensing, as even small microcolonies may induce gene expression since the signalling compounds may be concentrated within the microcolony and are not degraded (McLean *et al.*, 1997).

In Gram-positive bacteria, quorum sensing uses peptides (oligopeptides) as signals that are the ligands of membrane receptors (histidine kinases) or that interact with other proteins which in turn regulate the activity of the response regulator of signal transduction pathways (Miller and Bassler, 2001). For instance, in *Staphylococcus aureus*, the *agr* locus is responsible for controlling virulence gene expression via quorum sensing. At low cell density, the bacteria express protein factors that promote attachment and colonization, whereas at high cell density, the bacteria repress these traits and initiate secretion of toxins and proteases that are presumably required for dissemination (Lyon and Novick, 2004).

Quorum sensing may give biofilms their distinct properties. For example, expression of genes for antibiotic resistance at high cell densities may provide protection. Quorum sensing also has the potential to influence community structure by encouraging the growth of species beneficial to the biofilm. It is also possible that the physiological properties of bacteria in the community or biofilm

may be altered through quorum sensing (Kjelleberg and Molin, 2002). Novel antimicrobial strategies could be designed based on information garnered from studies of quorum sensing, which suggests that research on quorum sensing could have enormous practical applications (Davies *et al.*, 1998; Finch *et al.*, 1998).

5.1 Quorum sensing in *Candida* by farnesol

In *C. albicans* the ability to switch between yeast, pseudohypha, and hypha is an important characteristic. This morphological transition appears to be integrated with different types of environmental signal to assess whether conditions are the most favourable for growth in a specific cellular morphology (Odds, 1988; Brown and Gow, 1999). For example, *C. albicans* can be converted from a cellular yeast to a filamentous form *in vitro* by exposure to serum or N-acetylglucosamine (Niimi *et al.*, 1997). Moreover, the switching between different morphologies depends on the cells' metabolism and physiology, as well as division and polarity. For this reason, cells must communicate with each other as communities (Mitchell, 1998; Sanchez-Martinez and Perez-Martin, 2001).

Recently, a quorum sensing system was reported in *C. albicans* that is mediated by farnesol ($C_{15}H_{26}O$; molecular weight, 222; Hornby *et al.*, 2001; Fig 2A). This was the first study to identify an extracellular molecule which mediates a eukaryotic quorum-sensing system and which can be removed from cells of *C. albicans* by washing. Farnesol could block the induction of germ tubes by three chemically distinct triggers (L-proline, N-acetylglucosamine, and serum) preventing the yeast-to-mycelium conversion. It was also shown that farnesol alters cell morphology but does not alter growth rate (Hornby *et al.*, 2001). However, the farnesol concentration needed to inhibit germ-tube formation varied

depending on the conditions used. For instance, a low farnesol concentration (1-2 μM) was needed to inhibit germ-tube formation in defined medium, while a higher farnesol concentration (150 and 250 μM) was required when germ-tube formation was performed in 10 and 20% serum (Mosel *et al.*, 2005).

During chlamydospore morphogenesis, cells switch to filamentous growth and then develop elongated suspensor cells that give rise to chlamydospores. These chlamydospore cells are distinct from true hyphae in that they are wider and are not inhibited by the quorum-sensing factor farnesol. Instead, farnesol increases chlamydospore production, indicating that farnesol can also have a positive role (Martin *et al.*, 2005). Farnesol could also play an important role in protecting *C. albicans* cells from oxidative stress. Westwater *et al.* (2005) demonstrated that conditioned medium from a *C. albicans* stationary-phase culture is able to protect yeast cells from both hydrogen peroxide and superoxide anion-generating agents. Exponential-phase yeast cells preexposed to conditioned medium or culture with added exogenous farnesol were able to survive levels of oxidative stress that would normally kill actively growing yeast cells (Westwater *et al.*, 2005).

Another study by Oh *et al.* (2001) involved the synthesis and evaluation of farnesoic acid derivatives as morphogenic regulatory agents. This group found that the substance capable of regulating the yeast-to-hyphal morphological transition is farnesoic acid. However, farnesol was more active than farnesoic acid as an inhibitor of this transition, but was less selective, because it affected yeast cell growth too (Oh *et al.*, 2001).

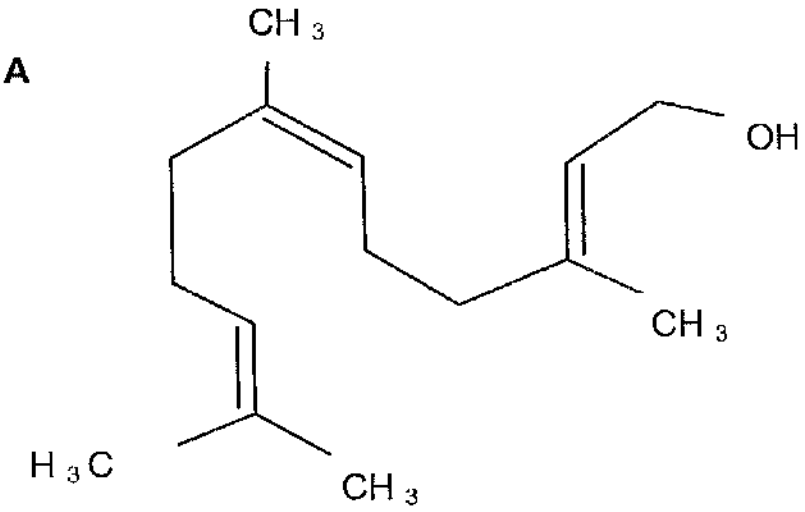
Figure 2

Quorum sensing molecules in *C. albicans*

A. Farnesol (a C_{15} isoprenoid alcohol) inhibits hyphal production

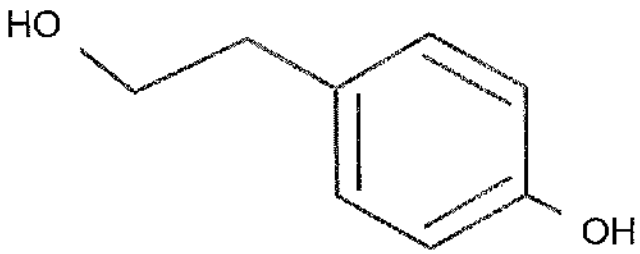
B. Tyrosol (2-[4-hydroxy phenethyl] ethanol) promotes hyphal production

A



Farnesol

B



Tyrosol

It was subsequently found that this observation was obtained with only one *C. albicans* strain (10231) which produces farnesoic acid and does not produces farnesol (Hornby and Nickerson, 2004). Unlike farnesoic acid which does not affect the growth of *C. albicans* but inhibits germ-tube formation, farnesoic acid derivatives inhibit germ-tube formation as well as growth of *C. albicans* (Kim *et al.*, 2002b). Another study demonstrated that protein-farnesyl transferase inhibitors inhibit germ-tube formation in serum but do not affect the growth of the yeast form of *C. albicans* (McGeady *et al.*, 2002).

Subsequent studies showed that out of forty natural and synthetic farnesol analogues, farnesol was the most active at inhibiting germ-tube formation by *C. albicans*, although farnesol from different sources showed different activity against germ-tube formation (Shchepin *et al.*, 2003). Moreover, fluorescent farnesol analogues were developed and thought to be constructive tools to study farnesol binding proteins and farnesol pharmacokinetics (Shchepin *et al.*, 2005). Additionally, it was found that farnesol production by *C. albicans* could be stimulated up to 8-fold by treating planktonic cells with sterol biosynthetic inhibitors like zaragozic acid and azole antifungal agents (Hornby *et al.*, 2003; Hornby and Nickerson, 2004).

Farnesol activity has been described in other fungi. For example, farnesol-treated cells of *S. cerevisiae* were characterized by a transition from budded to unbudded cells with significant loss of intracellular diacylglycerol (DAG). Moreover, it was found that this farnesol effect could be abolished by addition of a DAG analogue such as 1-oleoyl-2-acetyl-sn-glycerol (OAG) (Machida *et al.*, 1999). On the other hand, farnesol did not produce a morphological change in the

dimorphic fungus, *Ceratocystis ulmi*, which causes Dutch elm disease (Hornby *et al.*, 2004).

5.1.1 Farnesol and biofilms

C. albicans biofilm growth was inhibited in the presence of farnesol added at an early stage, and it was shown that farnesol inhibition was time-dependent. For example, preincubation with 300 μM farnesol completely inhibited biofilm formation whereas adding farnesol at the same concentration after 2 h of adherence time did not affect biofilm formation (Ramage *et al.*, 2002a). This finding was similar to the effect of farnesol on germ-tube formation. When farnesol was added at time zero, yeast cells failed to produce germ tubes. However, adding farnesol to 90-min mature germ tubes did not block germ-tube elongation (Mosel *et al.*, 2005). Supernatants from mature biofilms inhibited the filamentation of planktonic cells grown on RPMI medium, indicating that farnesol (or possibly other, similar quorum sensing molecules) is produced *in situ* in biofilms (Ramage *et al.*, 2002a). A recent report demonstrated the effect of farnesol on biofilm formation by non-*C. albicans* species. Farnesol inhibited biofilm formation by *C. parapsilosis* with a crepe, concentric or crater phenotype, but did not inhibit biofilm formation by *C. parapsilosis* with a smooth phenotype (Laffey and Butler, 2005).

Another interesting observation comes from studies on the effect of farnesol on a *C. albicans* mutant lacking Chk1p which was found to be avirulent in a murine model of haematogenously disseminated candidosis (Calera *et al.*, 1999). Germ-tube formation and biofilm formation by the null mutant (*chk1/chk1*) were not responsive to farnesol, unlike the wild-type strain (Kruppa *et al.*, 2004). The

CHK1 gene encodes a histidine kinase signal transduction protein, but the specific function of Chk1p has not been established (Kruppa *et al.*, 2003).

Analysis of gene expression by *C. albicans* biofilms exposed to farnesol using cDNA microarray analysis indicated that several genes were differentially expressed in the presence or absence of farnesol, including genes involved in hyphal formation, cell surface hydrophobicity, and drug resistance. For example, genes involved in hyphal formation such as *TUP1* were up-regulated, while *PDE2* (a nucleotide phosphodiesterase gene), along with the cell surface hydrophobicity gene *CSH1* and drug resistance genes *CDR1*, *CDR2* and *MDR1*, were down-regulated (Cao *et al.*, 2005).

5.1.2 Quorum sensing in fungal-bacterial biofilms

Fungal-bacterial interactions are common among microbial communities. Recently, interactions between two opportunistic pathogens, *C. albicans* and *P. aeruginosa*, were investigated in detail. Upon mixing cultures of *P. aeruginosa* and *C. albicans* it was observed that *P. aeruginosa* was able to attach to filaments of *C. albicans*, but almost never adhered to yeast-form *C. albicans* cells, even after prolonged incubation. Moreover, it was found that more bacteria attached to filaments after a short period of coincubation when *P. aeruginosa* cells were taken from stationary-phase rather than from exponential-phase cultures. After incubation of the mixed culture for 24h, *P. aeruginosa* formed biofilms on *C. albicans* filaments and killed the fungus. On other hand, *P. aeruginosa* did not bind or kill the yeast form of *C. albicans* (Hogan and Kolter, 2002).

Subsequent studies showed that this interaction between *C. albicans* and *P. aeruginosa* is dependent on *P. aeruginosa* quorum sensing molecules. It was demonstrated that 3-oxo-C_{12} homoserine lactone secreted by *P. aeruginosa* inhibits *C. albicans* filamentation without affecting *C. albicans* growth rates at levels similar those of farnesol. Moreover, structurally related compounds with a 12-carbon chain length such as C_{12}-acyl homoserine lactone and dodecanol also affected *C. albicans* filamentation. On other hand, other acylated homoserine lactones of different chain lengths did not affect *C. albicans* morphology (Hogan *et al.*, 2004).

Other studies suggested that farnesol might be useful as a new safe method for the treatment of atopic dermatitis infection caused by *S. aureus*, because farnesol showed an inhibitory effect on biofilm formation by *S. aureus* (Masako *et al.*, 2005a; Masako *et al.*, 2005b). Farnesol also shows modest antibacterial activity against biofilms of *Streptococcus sobrinus* (Koo *et al.*, 2002). However the mechanism of farnesol action remains unclear, since few studies of farnesol antibacterial activity have been undertaken so far.

Recently, farnesol has been tested for anticancer activity *in vitro* and *in vivo* in a number of animal models. The results of these investigations demonstrate significant chemotherapeutic properties (Burke *et al.*, 2002; Duncan and Archer, 2006). Farnesol is found in fruits and vegetables (Tatman and Mo, 2002) but farnesol-related toxicity has yet to be investigated in detail.

5.2 Quorum sensing in *Candida* by tyrosol

Tyrosol, a derivative of tyrosine, (Fig. 2B), has also been identified as a quorum sensing molecule in *C. albicans*. Chen *et al.* (2004) detected a signalling molecule in the culture medium of planktonically grown *C. albicans*, then showed that tyrosol was the active component, and was released into the medium continuously during growth. In addition, it was observed that accumulation of tyrosol in the medium increased along with increasing cell density. For example, tyrosol was detected after 10h of incubation, but accumulated to a concentration of 3 μM when the cell density was 1×10^{8} cells /ml. The authors also found that exogenous tyrosol added to the growth medium shortened the lag phase, and accelerated the conversion of yeast to filaments in germ-tube formation (Chen et al., 2004).

Earlier, tyrosol had been isolated from the culture medium of *Candida* species (Narayanan and Rao, 1976), and subsequent studies confirmed that tyrosol production by *C. albicans* and *C. tropicalis* was greater than that of other *Candida* species (Cremer *et al.*, 1999). Other investigations showed that different fungi, such as *S. cerevisiae* (Sentheshanmuganathan and Elsden, 1958; Batrakov *et al.*, 1993) and *Rhodiola* species (Xu *et al.*, 1998), as well as bacteria such as *Enterobacter cloacae,* can also produce tyrosol (Slininger *et al.*, 2004).

The possible role of tyrosol as a virulence factor for *C. albicans* and its interaction with host immune defences remain unknown. However, human neutrophil activity can be impaired by tyrosol produced by *Candida* species including *C. albicans* (Cremer *et al.*, 1999). Moreover, substantially higher concentrations of tyrosol are produced by *C. albicans* and *C. tropicalis* than by *C.*

parapsilosis and *C. glabrata*, suggesting a potential role in pathogenicity ranking (Cremer *et al.*, 1999).

6. Hormones and *Candida*

6.1 Prostaglandins

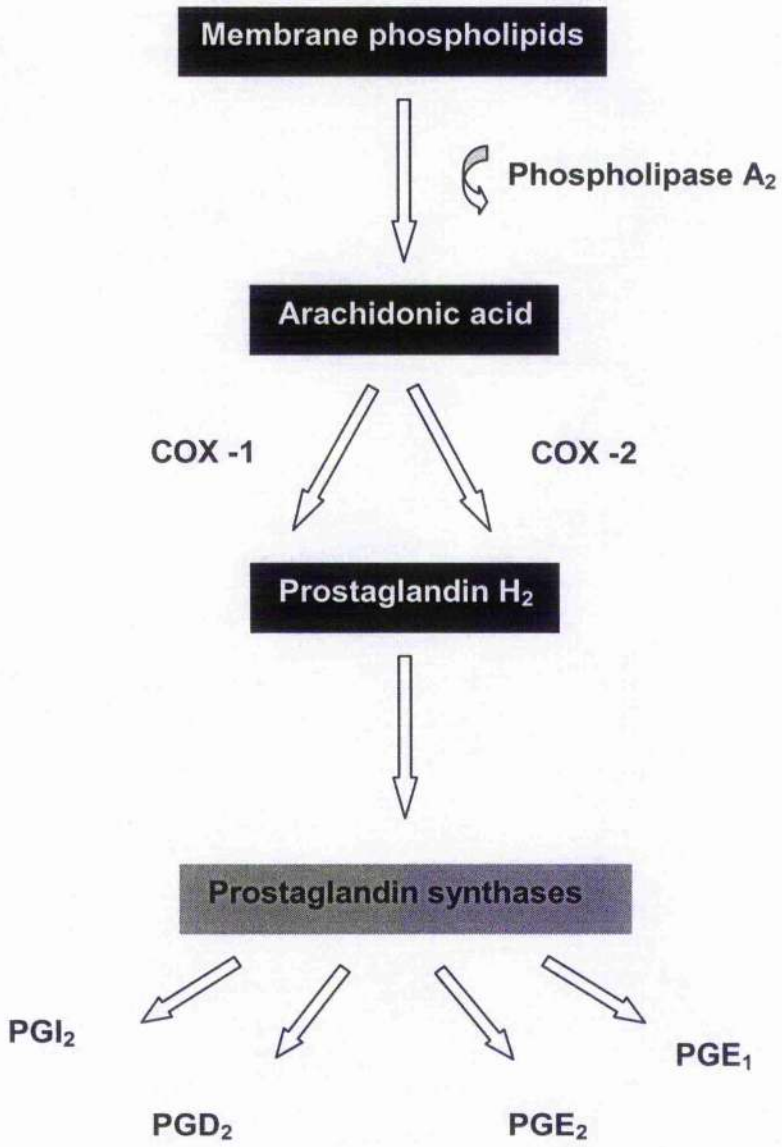
Prostaglandins are small lipid molecules that have diverse biological activities, including the modulation of host immune responses (Harris *et al.*, 2002). They are now known to be produced by pathogenic fungi as well as by mammalian cells (Noverr *et al.*, 2001; Noverr *et al.*, 2002; Noverr *et al.*, 2003). Both *C. albicans* and *Cryptococcus neoformans* secrete prostaglandins *de novo* or via conversion of exogenous arachidonic acid (Noverr *et al.*, 2001).

In mammalian systems, arachidonic acid, formed by cleavage of phospholipids, is converted to prostaglandin H_2 (PGH_2) by the cyclooxygenase isoenzymes, COX-1 and COX-2 (Fig. 3). It is thought that COX-1 is expressed constitutively in most tissues of the body and is responsible for a basic level of PGH_2, whereas COX-2 is mainly an inducible enzyme involved in the regulation of inflammation (Dannhardt and Kiefer, 2001). Prostaglandin synthases subsequently convert PGH_2 into a series of prostaglandins, including PGI_2, PGF_2, PGD_2 and PGE_2 (Harris *et al.*, 2002). A PGE series lipid, possibly PGE_2, was purified from both *C. albicans* and *C. neoformans*. It enhanced germ-tube formation by *C. albicans* and was also biologically active on mammalian cells (Noverr *et al.*, 2001).

Figure 3

Synthesis of prostaglandins

The production of prostaglandins begins with the liberation of arachidonic acid from membrane phospholipids by phospholipase A_2. Arachidonic acid is converted to PGH_2 by the cyclooxygenase enzymes COX-1 and COX-2. Different synthase enzymes then convert PGH_2 to the whole family of prostaglandins.


Membrane phospholipids
Phospholipase A_2
Arachidonic acid
COX -1
COX -2
Prostaglandin H_2
Prostaglandin synthases
PGI_2
PGD_2
PGE_2
PGE_1

The role of prostaglandins in fungal biology is unknown. They may function as regulators of gene expression, as in animal cells. However, prostaglandin production could also represent an important virulence factor by promoting fungal colonization and chronic infection. Despite the fact that fungal pathogens produce prostaglandins, live *C. albicans* cells can cause significant endothelial release of eicosanoids, mainly prostaglandins. In addition, the ability of different *Candida* species to induce endothelial prostaglandin release is closely related to their capacity to injure the endothelium. *C. albicans* was the only *Candida* species that either stimulated prostaglandin release or damaged endothelial cells (Filler *et al.*, 1991). Subsequent studies demonstrated that endothelial cell invasion by *C. albicans* appeared to stimulate the conversion of arachidonic acid into prostaglandins by upregulating the synthesis of endothelial cell cyclooxygenase and increasing the activity of the endothelial cell phospholipase (Filler *et al.*, 1994).

6.2 COX inhibitors

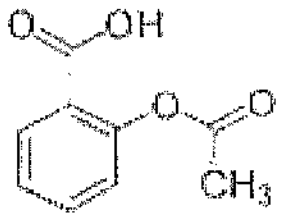
Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used therapeutics, primarily for the treatment of pain and inflammation, especially arthritis. These drugs specifically block the biosynthesis of mammalian prostaglandins by inhibiting one or both of the cyclooxygenase isoenzymes, COX-1 and COX-2; therefore NSAIDs can be classified COX 1 and COX 2 inhibitors (Dannhardt and Kiefer, 2001; Fig. 4).

Figure 4

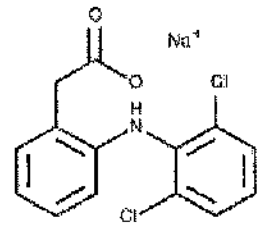
Structures of COX inhibitors

* COX-1 inhibitors

** COX-2 inhibitors

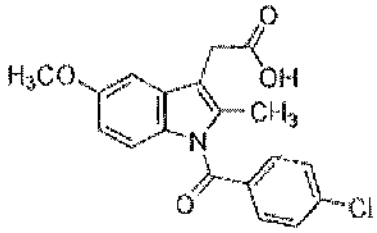


Aspirin *

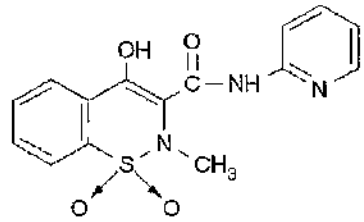


Diclofenac *

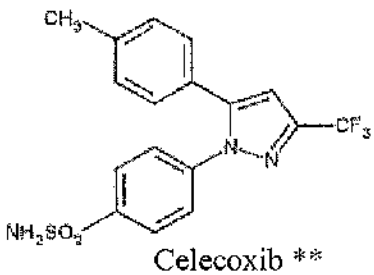
Ibuprofen *



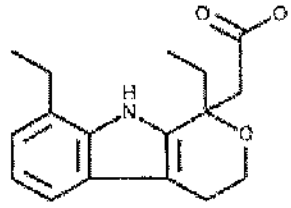
Indomethacin *



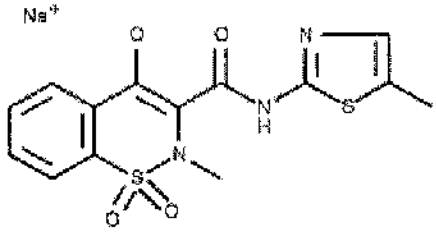
Piroxicam *



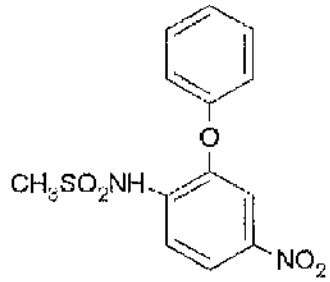
Celecoxib **



Etodolac **



Meloxicam **



Nimesulide **

6.2.1 COX inhibitors and antibacterial drugs

Although the normal usage of COX inhibitors is as anti-inflammatory drugs, several investigations have evaluated possible improvements in antibiotic therapy by co-administration of COX inhibitors such as sodium salicylate. For example, salicylate can have both beneficial and detrimental effects on the growth of bacteria, including the induction of multiple antibiotic resistance with some organisms and a reduction in drug resistance with others (Price *et al.*, 2000). *Serratia marcescens* resistance to ciprofloxacin and nalidixic acid was enhanced in the presence of salicylate (Berlanga and Vinas, 2000), whereas treatment of an invasive experimental *S. aureus* infection (endocarditis) with intravenous aspirin caused a significant reduction in bacterial densities within target tissues (vegetations and kidneys) (Kupferwasser *et al.*, 1999).

Subsequent studies suggested that aspirin may exert a direct effect on bacterial adhesins that are necessary for *S. aureus* colonization and propagation in host tissues (Kupferwasser *et al.*, 2003). Additionally, Nicolau *et al.* (1995) demonstrated a combined effect of aspirin and vancomycin using a rabbit endocarditis model; they found a significant reduction in *S. aureus* densities within target tissues when aspirin was given prior to and during vancomycin therapy (Nicolau *et al.*, 1995). These results indicate that a combination of vancomycin and aspirin could be useful for treatment of *S. aureus* infections *in vivo*. Another interesting investigation showed that aspirin alone inhibits the growth of *Helicobacter pylori* and enhances the activity of some widely used antibiotics, such as amoxicillin, metronidazole and clarithromycin (Wang *et al.*, 2003).

6.2.2 COX inhibitors and antifungal drugs

The viabilities of *C. albicans* and *C. neoformans* were reduced significantly in the presence of some COX inhibitors suggesting that an essential cyclooxygenase enzyme might be responsible for fungal prostaglandin synthesis (Noverr *et al.*, 2001). Moreover, other fungi similar to dematophytes were remarkably susceptible to flurbiprofen which is a NSAID (Chowdhury *et al.*, 2003). Additionally it has been suggested that aspirin inhibits *C. albicans* by inhibition of *Candida* oxylipins, which are similar to prostaglandins (Deva *et al.*, 2001).

Fluconazole is widely used in the treatment of *Candida* infections. However, fluconazole-resistant strains frequently appear, and therefore antifungal agents particularly fluconazole, have been investigated in combination with other drugs as a possible new therapeutic strategy. In the last few years several *in vitro* studies have evaluated the effect of fluconazole in combination with ibuprofen against *C. albicans*. For example, Scott *et al.* (1995) demonstrated the synergistic effect of fluconazole in combination with ibuprofen. Moreover, the MICs of fluconazole for some fluconazole-resistant strains decreased 2 to 128-fold when the drug was associated with ibuprofen (Pina-Vaz *et al.*, 2000). Another report showed that there was no synergistic effect of fluconazole combined with ibuprofen against fluconazole-susceptible strains, but a remarkable effect was seen against fluconazole-resistant strains (Arai *et al.*, 2005). Subsequent studies showed that resistance related to efflux pumps which export drugs in fluconazole-resistant *C. albicans* strains could be reversed by pre-treating the cells with ibuprofen (Pina-Vaz *et al.*, 2005).

6.3 Steroid hormones

Hormones are biochemical signalling molecules that regulate physiological events in multicellular organisms. However, similar compounds have been described in unicellular organisms. For instance, fungi have similar systems of chemical communication including fungal hormones or pheromones (Gooday and Adams, 1993). These include sirenin hormones, which have precise functions and control sexual reproduction in *Allomyces macrogynus*. Similarly, a and α mating factors are the sole primary signals for inducing mating in *S. cerevisiae* (Gooday and Adams, 1993). Furthermore, fungi appear to interact with mammalian hormones at a level closely analogous to the interaction of mammalian cells with these chemical molecules.

Loose *et al* (1981) described a cytosolic protein from yeast cells of *C. albicans* that bound [^{3}H] corticosterone and [^{3}H] progesterone with high affinity and selectivity. They also demonstrated that a crude ethanolic extract of yeast cells of *C. albicans* contained a factor which competed reversibly with [^{3}H] corticosterone for the fungal binding site. This material was released into the growth medium. Loose *et al* (1981) concluded that the ethanolic extract of *C. albicans* contained an endogenous ligand for the cytosol binder and proposed that *C. albicans* possesses both components of a hormone receptor system, namely a ligand and a binding protein. (Loose *et al.*, 1981).

Subsequently White and Larsen (1997) investigated the role of the mammalian hormone, estrogen, in initiating the transformation of *C. albicans* from yeast to mycelial growth, which they believed to be associated with the organism's virulence. They found that the percentage of yeast cells germinating

was profoundly reduced in stripped serum (without estrogen) compared to unstripped serum (White and Larsen, 1997).

Deslauriers *et al.* (1995) observed that topical application of corticosteroids to the oral mucosa of 75 mice increased the residual *Candida* population. For example, topical application of a corticosteroid increased the *Candida* population on day 4 by up to 40-fold, and by day 21 the population was 400-fold greater. In contrast, on cessation of treatment, normal levels of *Candida* were quickly restored. Their results suggest that topical application of corticosteroids may dramatically shift the host-parasite relationship in favour of *Candida* (Deslauriers *et al.*, 1995).

Aims and Objectives of Research

Bacterial biofilms have been studied extensively over the past 25 years, and much is known about their development, including cell signalling and regulation within the biofilm via quorum sensing. In 2001, farnesol, a specific signalling molecule was detected in culture supernatants of planktonically grown *C. albicans* and shown to prevent the formation of mycelia. A subsequent brief report showed that farnesol has an inhibitory effect on *C. albicans* biofilms but the role of farnesol in biofilm development was not fully investigated. More recently, tyrosol was also identified as quorum sensing molecule which could stimulate mycelial development in *C. albicans*; however, tyrosol synthesis in *C. albicans* biofilms was not investigated. Prostaglandins, which are potent regulators of host immune responses, are now known to be produced by pathogenic yeasts, including *C. albicans*, but the possible role of prostaglandins in biofilm development and morphogenesis is still unknown.

The aim of this project was to explore the roles of farnesol, tyrosol and prostaglandin(s) in germ-tube formation and biofilm development by different strains and mutants of *C. albicans*.

Specific objectives included the following:

1) Evaluation of germ-tube production and biofilm formation by a variety of genetically engineered mutants of *C. albicans*.

2) Determination of the effect of farnesol and/or tyrosol on germ-tube formation and biofilm development.

3) Investigation of the structure of biofilms formed in the presence or absence of farnesol, tyrosol and prostaglandin.

4) Measurement of tyrosol production by HPLC during planktonic growth and biofilm formation by *C. albicans*, including strains defective in germ-tube formation.

5) Investigation of the effects of COX inhibitors, prostaglandins and a combination of COX inhibitors with antifungal agents on biofilm development.

6) Measurement of prostaglandin production during planktonic growth and biofilm formation in the presence or absence of COX inhibitors using an ELISA assay.

7) Determination of the effect of hormones and steroids on the development of *Candida* biofilms and on germ-tube formation.

MATERIALS AND METHODS

1. Organisms

Twentyfour *C. albicans* strains were used in this study, together with one strain of *C. glabrata* (Table 1).

2. Growth Media

Full details of all media are given in Appendix 1.

2.1 Yeast nitrogen base

Unless otherwise stated, yeast nitrogen base (YNB; Difco) supplemented with 50 mM glucose was used as the standard liquid medium for growth of planktonic cells and biofilms of *C. albicans*. One litre of this medium contained 6.7g of yeast nitrogen base and 9g of glucose (50 mM), with a final pH of 5.4 ± 0.1; it was autoclaved at 10 p.s.i for 10 min.

2.2 Yeast nitrogen base plus uridine

YNB medium, prepared as described earlier, was supplemented with filter-sterilized uridine solution, (40 mg / litre, final concentration; Sigma) for growth of the Nebraska strains as planktonic cells or biofilms.

2.3 Yeast nitrogen base plus 17 mM acetic acid

YNB medium, prepared as described earlier, was supplemented with acetic acid (1 g / litre), adjusted to pH 4.2 and filter-sterilized. This medium was used to evaluate the combined effects of aspirin and fluconazole against mature biofilms of *C. albicans*.

Table 1. Organisms used in this study

Strain	Description
C. albicans GDH 2346	**Isolated at Glasgow Dental Hospital from a patient with denture stomatitis**
C. albicans CAI-4	Wild-type strain
C. albicans H48	Hairy colony morphology
C. albicans H51	Hairy
C. albicans H147	Hairy - wrinkled
C. albicans H121	Hairy - wrinkled
C. albicans H1	Mountain-like
C. albicans H253	Hairy - wrinkled **Strains from University of Nebraska (Jacob Hornby) including six mutants with altered colony morphology and response to farnesol (Jensen *et al.*, 2006). All strains were ura$^-$.**
C. albicans SC5314	Wild-type strain
C. albicans JKC19	Morphological mutant blocked in signalling pathway (*cph1/cph1*)
C. albicans HLC52	Morphological mutant blocked in signalling pathway (*efg1/efg1*)
C. albicans HLC54	Morphological mutant blocked in two signalling pathways (*cph1/cph1 efg1/efg1*) **Strains from University of Aberdeen (Neil Gow) including three morphological mutants.**
C. albicans DAY185	Wild-type strain
C. albicans DAY25	Mutant blocked in signalling pathways (*rim 101/rim 101*)
C. albicans VIC25	Mutant blocked in signalling pathways (*mds3/mds3*)
C. albicans VIC28	Mutant blocked two signalling pathways (*rim 101/rim 101 mds3/mds3*) **Strains from Columbia University (Aaron Mitchell) including three mutants in pH-response or virulence.**
C. glabrata AAHB 12	**Isolated from patient with line infection at Crosshouse Hospital, Kilmarnock.**

Table 1 (Continued)

C. albicans CAF2-1	Wild-type strain
C. albicans N	Mutant blocked in signalling pathway (*nik1/nik1*)
C. albicans Ssk21	Mutant blocked in signalling pathway (*ssk1/ssk1*)
C. albicans Chk21	Mutant blocked in signalling pathway (*chk1/chk1*)
C. albicans Chk23	Mutant blocked in signalling pathway (*chk1/CHK1*)
C. albicans N2	Mutant blocked in signalling pathway (*skn7/skn7*)
C. albicans R15	Mutant blocked in signalling pathway (*skn7/SKN7*)
C. albicans SN	Mutant blocked in signalling pathway (*sln1/sln1 nik1/NIK1*)
	Strains from Georgetown University (Bastlaan Krom) (Yamada-Okabe *et al.*, 1999; Kruppa *et al.*, 2003).

All strains were maintained on slopes of Sabouraud dextrose agar (Difco) and subcultured monthly. Every 2 months, cultures were replaced by new ones freshly grown from freeze-dried stocks. Freeze-dried yeasts were maintained in a dried state in small evacuated glass ampoules kept at -20 °C.

2.4 Sabouraud dextrose agar

Sabouraud dextrose agar (Oxoid; 65g/litre) was autoclaved at 121 °C for 15 min. The final pH was 5.6 ± 0.2. After autoclaving, the medium was cooled to 50 °C, and then dispensed in petri dishes or universals for plate and slope cultures, respectively. This medium was used to maintain all *C. albicans* strains as well as *C. glabrata* AAHB 12.

2.5 Sabouraud dextrose broth

Sabouraud dextrose broth was prepared using 40g/litre dextrose (Difco) and 10g peptone (Duchefa). The medium was autoclaved at 121 °C for 15 min. This medium was used to examine prostaglandin production by planktonic cells of *C. albicans* and to evaluate the combined effect of some COX inhibitors and antifungal agents.

2.6 RPMI 1640 buffered with MOPS

RPMI 1640 (with L-glutamine) liquid medium (Cambrex) was buffered with MOPS [3-(N-morpholino) propanesulfonic acid; 34.53g /litre] to a final pH of 7.0 at 25°C (NCCLS, 1995). The medium was then filter sterilized (filter pore size, 0.2 μm; Sartorius Minisart) and stored at 4 °C. This medium was used to evaluate the combined effect of COX inhibitors and some antifungal agents.

2.7 RPMI 1640 buffered with HEPES

RPMI 1640 (with L-glutamine) liquid medium (Cambrex) was buffered with HEPS [4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt; 16.4 g/litre] to a final pH of 7.0 at 25°C (NCCLS, 1995). The medium was then filter sterilized (filter pore size, 0.2 μm; Sartorius Minisart) and stored at 4 °C.

This medium was used to evaluate the combined effect of COX inhibitors and some antifungal agents against *Candida* biofilms.

2.8 Yeast peptone dextrose

Yeast peptone dextrose (YPD) medium consisted of yeast extract (10g/litre), peptone (20g/litre) and dextrose (20g/litre), adjusted to pH 6. This medium was used to grow inocula (overnight cultures) for biofilm formation in RPMI 1640 in 96-well microtitre plates (Ramage *et al.*, 2002a).

2.9 Vagina-simulative medium

Vagina-simulative medium, pH 4.2, was used to determine the combined effect of aspirin and fluconazole on *C. albicans* biofilms. This medium was originally developed to imitate human vaginal fluid (Owen and Katz, 1999). It was sterilized by filtration (0.2 µm pore size).

2.10 Hornby medium

Hornby medium was prepared and sterilized by filtration (0.2 µm pore size). This medium was used to stimulate germ-tube formation by *C. albicans* as described by Hornby *et al.* (2001).

2.11 Germ-tube inducers

Different inducers were used to examine germ-tube formation by *C. albicans*. They were dissolved in 50 mM potassium phosphate buffer (pH 6.5). The inducers were:

a) 10 mM proline (Sigma)

b) 2.5 mM N-acetylglucosamine (Sigma)

c) 10 mM proline + 2.5 mM N-acetylglucosamine

d) 5% foetal bovine serum (Gibco)

3. Chemicals

3.1 COX inhibitors and PGE_2

Stock solutions (100 mM) of diclofenac, ibuprofen, indomethacin, meloxicam, piroxicam, etodolac, celecoxib and nimesulide (Sigma) were prepared in dimethyl sulfoxide (DMSO). Stock solutions (100 mM) of aspirin (acetylsalicylic acid; Sigma) and salicylic acid (Sigma) were prepared in ethanol. Stock solutions (100 μM) of sodium salicylate (Sigma) were prepared in water. All stock solutions were filter-sterilized. In biofilm experiments, COX inhibitors were used at final concentrations ranging from 10 μM to 1 mM. PGE_2 (Cayman Chemicals, Ann Arbor, Michigan) was dissolved in ethanol and used at final concentrations of 10 nM, 100 nM and 1 μM.

3.2 Steroids

Stock solutions (100 mM) of progesterone, corticosterone, dexamethasone, prednisolone, hydrocortisone and β-estradiol (Sigma) were prepared in ethanol. Steroids were used at final concentrations ranging from 0.01 μM to 1 mM.

3.3 Quorum sensing molecules

3.3.1 Farnesol

Stock solutions of farnesol (100 mM; trans, trans-farnesol; Sigma) were prepared in methanol and filter-sterilized. For biofilm experiments, farnesol was added at the beginning of the adhesion period, or throughout adhesion and again at time zero of biofilm formation.

3.3.2 Tyrosol

Stock solutions (100 mM) of tyrosol [2-(4-hydroxyphenyl) ethanol; Aldrich, Germany] were prepared in water, filter-sterilized, and stored at 4°C.

3.4 Propranolol

Stock solutions (100 mM) of propranolol (Sigma) were prepared in water, filter-sterilized, and stored at 4°C.

3.5 1-Oleoyl-2-acetyl-sn-glycerol (OAG)

Stock solutions (100 mM) of 1-oleoyl-2-acetyl-sn-glycerol (OAG) (Sigma) were prepared in dimethyl sulfoxide (DMSO), filter-sterilized, and stored at 4°C.

3.6 Dibutyryl cyclic-AMP

Stock solutions (100 mM) of N^6,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate (dibutyryl cAMP) (Sigma) were prepared in water, filter-sterilized, and stored at 4°C.

3.7 Antifungal agents

3.7.1 Amphotericin B

Stock solutions of amphotericin B (1600 µg/ml) were prepared DMSO, filter-sterilized and stored at -20 °C. Amphotericin B is light sensitive and therefore vials were covered in foil for protection from the light.

3.7.2 Fluconazole

Stock solutions of fluconazole (1600 µg/ml) were prepared in water, filter-sterilized, and stored at -20 °C.

4. Biofilm formation on catheter disks

4.1 Preparation of catheter disks

Biofilms were grown on small disks (surface area, 0.5 cm^2; Fig. 5 A) cut by a metal punch (Fig. 5 B) from polyvinyl chloride (PVC) Faucher tubes (French gauge 36; Vygon, Cirencester, UK), or from polystyrene 75 cm^2 tissue culture flasks (Costar; Corning Incorporated, USA). Disks were placed in petri dishes and sterilized by exposure to ultraviolet radiation for 20-30 min on each side.

4.2 Preparation of microorganisms

All organisms were grown in YNB medium containing 50 mM glucose unless stated otherwise. For the Nebraska strains only, uridine (40mg/litre) was added. Batches of medium (25 ml, in 100-ml Erlenmeyer flasks) were inoculated from fresh culture slopes and incubated at 37 °C in an orbital shaker at 60 rpm. Under these conditions all the strains grew exclusively in the budding-yeast phase. Cells were harvested after 24 h and washed twice in 0.15 M phosphate-buffered saline (PBS; pH 7.2; Sigma). Washed cell suspensions were adjusted to an optical density of 0.8 at 520 nm before use in biofilm experiments.

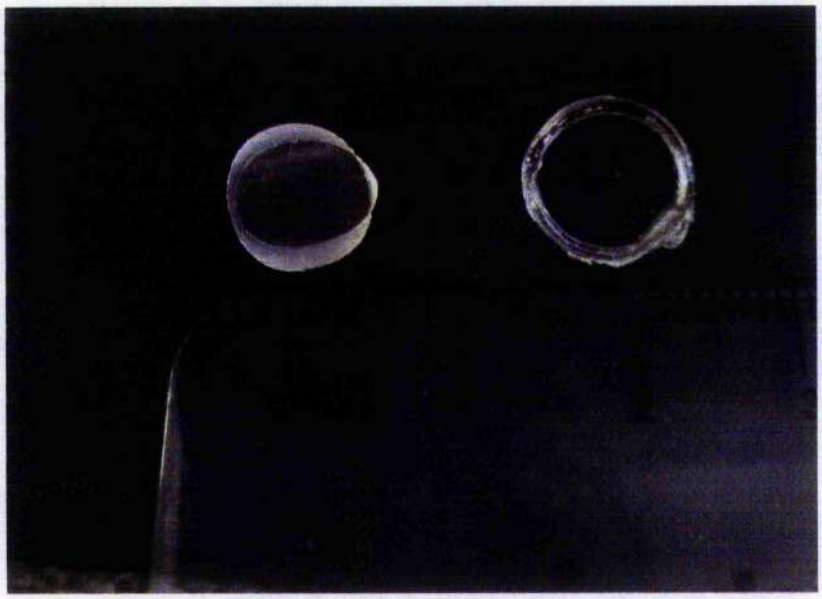
Figure 5

Biofilm disks and metal punch

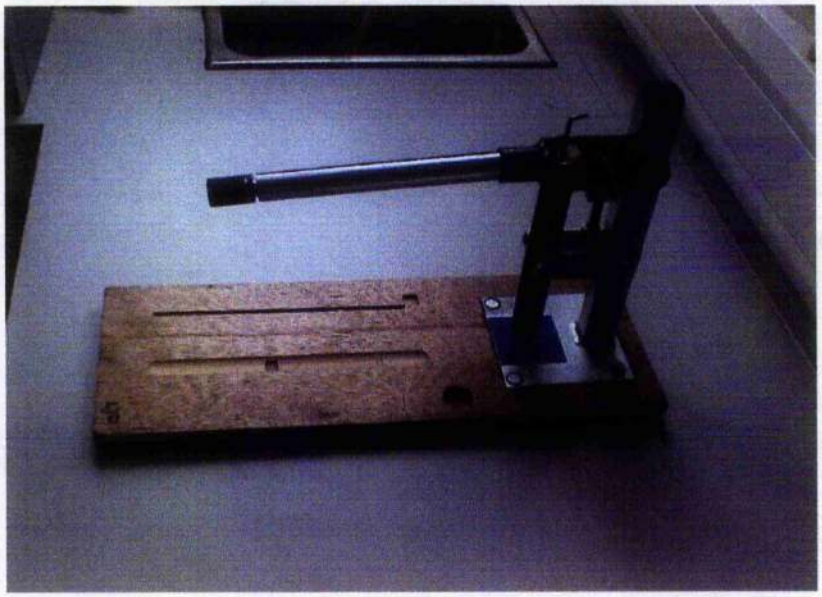
A) On the left, a polyvinyl chloride (PVC) disk cut from catheter tubing (French gauge 36; Vygon, Cirencester, UK). On the right, a polystyrene disk cut from 75 cm^2 tissue culture flask.

B) A metal punch used to cut PVC disks from catheter tubing.

A



B



4.3 Biofilm formation

Biofilms were grown on small disks (surface area, 0.5 cm^2) as described previously in Section 4.1. The disks were placed in the wells of 24-well Nunclon tissue culture plates, and a standardized cell suspension (80 µl) was applied to the surface of each one. Initially, incubation lasted for 1 h at 37 °C (adhesion period). Non-adherent organisms were removed by washing with PBS, and the disks were then incubated for a further 48 h at 37 °C, submerged in 1 ml of growth medium (biofilm formation). At the end of the incubation period, disks were washed gently twice with PBS and transferred to fresh 24-well plates for quantitative measurement of biofilm formation.

4.4 Quantitative measurement of biofilm formation

Biofilm growth was quantified colorimetrically by a 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay. XTT solution (250 µg / ml; Sigma) was prepared in PBS containing 1 % (wt/vol) glucose, and 1 ml was added to each well of fresh plates containing the biofilm disks. Menadione solution (Sigma; 1 mM in acetone; 4 µl) was also added to the wells and the plates were incubated for 5 h at 37 °C in the dark. After incubation, the liquid was removed from each well and transferred to a microfuge tube, clarified by centrifugation at 13,000 rpm, and XTT formazan production was measured at 492 nm. Control disks were used to check for interference with XTT reduction by any inhibitors or agents used in the experiment.

5. Biofilm formation in wells of microtitre plates

This assay was used to evaluate the effect of farnesol on biofilm formation on the surfaces of wells of microtitre plates. Batches of YPD medium (20 ml) in 100 ml flasks were inoculated with one loopful of *C. albicans*, from a slope culture. Inoculated flasks were incubated on an orbital shaker at 37 °C and 60 rpm overnight when the cells had reached the stationary phase of growth. Cells were harvested and washed twice in sterile PBS at 3000 rpm for 5 min. The cells were then resuspended in RPMI 1640 buffered with MOPS and adjusted to a density of 1.0×10^6 cells/ml in RPMI 1640 by counting in a haemocytometer. Standardized cell suspensions (100 µl) were added to wells of flat-bottomed 96-well plates (Iwaki Microplate, Japan); these suspensions were added with and without 1 mM farnesol. Plates were incubated at 37 °C for 48h. After biofilms were formed, the medium was aspirated and nonadherent cells were removed by thoroughly washing the biofilm three times in sterile PBS. XTT stock solution (0.5 mg/ml in PBS+1% glucose) was prepared and menadione (10 mM prepared in acetone) was added to a final concentration of 1 µM. A 100-µl aliquot of this XTT-menadione stock solution was added to each well containing a prewashed biofilm, and to control wells (to measure background XTT levels). Plates were incubated in the dark for 2 h at 37 °C, and the colorimetric change was measured in a microtitre plate reader at 490 nm.

6. Measurement of tyrosol in culture supernatants

6.1 Supernatant preparation

6.1.1 Growth of planktonic cells and biofilms

For planktonic cultures, standardized cell suspension (100 µl) was used to inoculate YNB medium (50 ml, in 250-ml Erlenmeyer flasks) giving a cell density of $4x10^4$ cells/ml by counting in a haemocytometer. Cultures were incubated at 37 °C in an orbital shaker at 60 rpm for time periods of up to 48 h and then centrifuged at 3000 rpm. The supernatants were decanted and filter-sterilized through a 0.2 µm filter. Biofilms were grown on the surface of 75-cm^2 flat-bottom tissue culture flasks (Costar; Corning Incorporated, USA) with vented cap. Standardized cell suspensions of *C. albicans* strains were prepared as described previously (Section 4.2). Cell suspension (10 ml) was added to the surface of each tissue culture flask, and the cells were allowed to adhere for 1 h at 37 °C. The remaining cell suspension was decanted and non-adherent cells were removed by washing with 10 ml of PBS. Fresh YNB (110 ml) was added and biofilms were formed over 48h at 37 °C under static conditions. The biofilm supernatant was then decanted, centrifuged at 3000 rpm and filter-sterilized using a 0.2 µm filter.

6.1.2 Dry weight determinations

At the end of the incubation period, 3 ml of planktonic cells or biofilm cells were collected on preweighed cellulose nitrate filters (Whatman; 0.45-µm pore size; 25-mm diameter) and cell dry weights determined as described in Section 11.

6.2 Tyrosol extraction and quantification using HPLC

A method for the extraction and quantification of tyrosol (2-(4-hydroxyphenyl) ethanol) from *C. albicans* culture supernatant was developed in collaboration with Mohammed Oteef (PhD student) and Dr. Hugh Flowers (Department of Chemistry, University of Glasgow). The method is based on the extraction of tyrosol using solid phase extraction (SPE). Tyrosol in the extract was quantified by a reverse phase (RP) HPLC method.

6.2.1 Chemicals

Tyrosol (2-(4-hydroxyphenyl) ethanol); 98% pure; Aldrich, Germany) was used to prepare a 1000 μM stock solution in acetonitrile which was then diluted to give working standards. Acetonitrile and methanol were HPLC Grade solvents (Fisher Scientific, UK). Sulphuric acid was AnalaR Grade (BDH, UK).

6.2.2 Solid phase extraction (SPE) method

Culture supernatant (50 ml) was acidified by the addition of 0.2 ml of 0.1 M sulphuric acid. A C-18 Waters Sep-Pak Plus (820 mg; Fig. 6A) cartridge was conditioned with 10 ml of methanol followed by 10 ml of 1.0 mM sulphuric acid. The acidified supernatant was then loaded on to the cartridge. Tyrosol was eluted with 10 ml of 7.5% acetonitrile in 1.0 mM sulphuric acid. The loading and eluting flow rates were controlled to be 2-4 ml/min in a SPE vacuum manifold (Fig. 6 B). No washing step was carried out as it caused a reduction in the recovery of tyrosol.

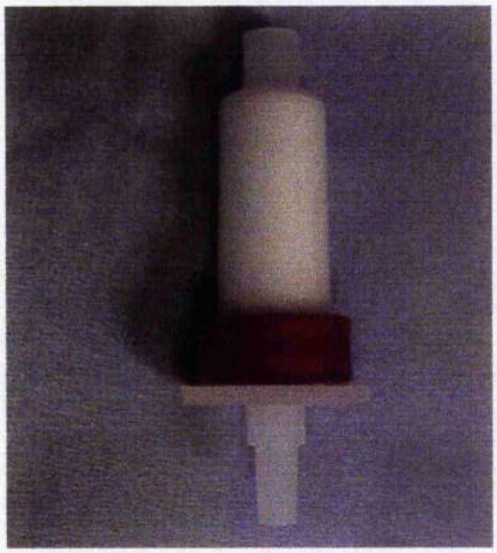
Figure 6

Sep-Pak cartridge and vacuum manifold

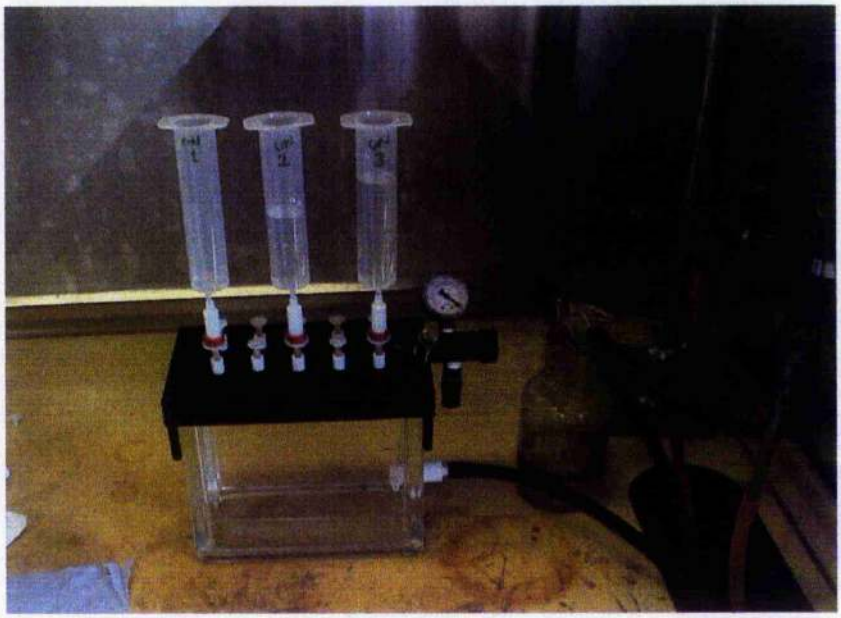
A) Waters Sep-Pak ® Plus Environmental C18 cartridge. The long body cartridge is packed with 820 Sep-Pak silica-based C18 bonded phase with sorbent particle size ranging from 55-105 μm and a pore size of 125Å. The carbon loading is 12% and the suitable pH range is 2-8.

B) The vacuum manifold used to control the flow rates during the solid phase extraction. The vacuum is generated by a water pump and controlled by a pressure valve to give the desired flow rate. The loading and cluting flow rates were controlled to be 2-4 ml/min.

A



B



6.2.3 HPLC equipment

The HPLC system used consisted of a Merck-Hitachi L-7100 pump (Fig. 7A), a L7200 autosampler and L4500 Diode Array Detector (Fig. 7B). The signal and UV spectra were processed by Merck-Hitachi Chromatography Data Station software.

6.2.4 Chromatographic conditions

HPLC analyses were carried out using a C-18 Waters Spherisorb ODS2 analytical column (4.6 x 250 mm). The elution profile consisted of three mobile phase compositions over 30 min of run-time. For the first 10 min, the mobile phase consisted of acetonitrile: 1.0 mM H_2SO_4 (10:90, v/v) for eluting tyrosol. The mobile phase composition for the second 10 min was acetonitrile: water (50:50 v/v) to clean up the column after some late eluted compounds. The column was then re-conditioned using acetonitrile: 1.0 mM H_2SO_4 (10:90, v/v) for 10 min to be ready for the next injection. The flow rate was set at 1.5 ml/min and the analysis was carried out at room temperature. An aliquot of 10 µl from the purified extract was injected into the HPLC system. The photodiode array was programmed to record data from 200-400 nm. The purity of tyrosol peaks was checked and the peaks were identified by comparing their spectra with those of a pure standard. The amount of tyrosol present was quantified by external calibration.

Figure 7

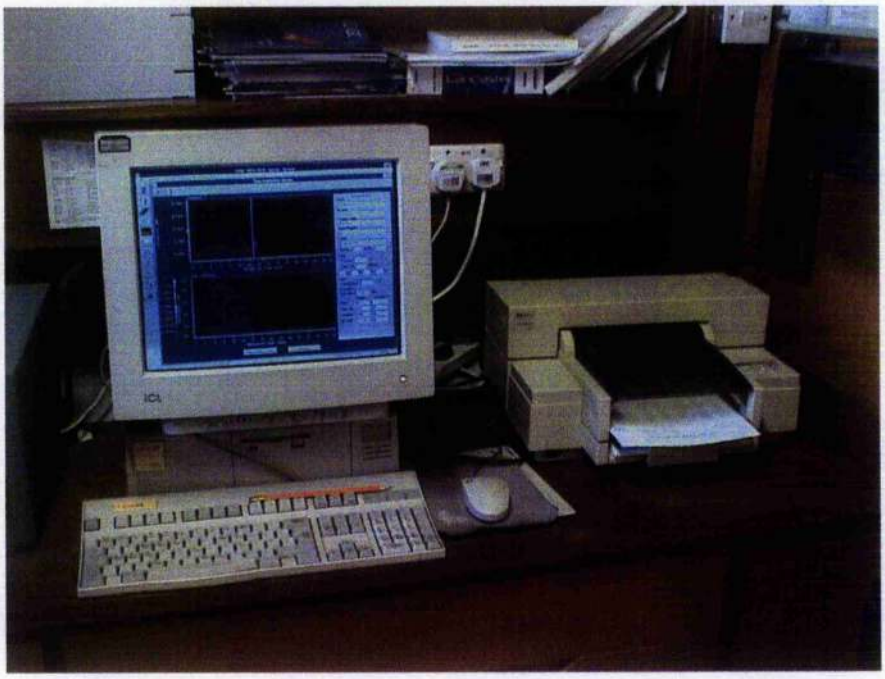
The HPLC system used for the separation and quantification of tyrosol

A) Merck-Hitachi L-7100 4 channels gradient pump (bottom left). It was programmed to deliver the mobile phase as follows: t=0 to 10 min, 10% acetonitrile: 90% 1.0 mM H_2SO_4; t=10.1 to 20 min, 50% acetonitrile: 50% H_2O; t=20.1 to 30 min, 10% acetonitrile: 90% 1.0 mM H_2SO_4. The flow rate was set at 1.5 ml/min. A L-7200 autosampler (top left) was used to inject 10 µl of sample via the injection port which is connected to a 100 µl loop. A L-4500 Diode Array Detector (bottom right) was programmed to record data from 200-400 nm for peak identification and purity checks.

B) The computer containing Merck-Hitachi Chromatography Data Station software which was used for signal and UV spectra processing.

A

B



6.2.5 Method validation

The linearity of the detector response was assessed by analysing tyrosol standards in the range 20-100 μM (Fig.8). The system precision was examined by analysing replicate injections (n =7) at the targeted limit of quantification (1 μM) and at the usual working level (50 μM). The recovery of the SPE method was assessed by spiking replicate samples with 6 μM tyrosol (Fig. 9A). The growth medium was analysed for possible interference, and was shown to have no interfering substances eluting at or near the retention time of tyrosol (Fig. 9B).

7. Germ-tube formation

7.1 Measurement of germ-tube formation

This assay was used to evaluate the activity of different germ-tube inducers on germ-tube formation. Initially, a 100-ml Erlenmeyer flask containing 25 ml YNB was inoculated from a slope culture and incubated at 37 °C in an orbital shaker operating at 60 rpm. After 24 h, cells were harvested and washed twice in 50 mM potassium phosphate buffer, pH 6.5. The washed cell suspension was adjusted to 2 $x10^9$ cells/ml in the same buffer. A portion (25 μl) of the adjusted inoculum was added to 5 ml of different germ-tube inducers as described in Section 2.11 to give a final cell density of 1 x 10^7 cells/ml. Flasks were incubated with gentle shaking at 37 °C for up to 5h. The suspensions were examined for the percentage of germ tubes present using a light microscope; 200 cells were counted each time.

Figure 8

Tyrosol calibration curve

The range examined was 0-100 μM. The detector response showed good linearity (R^2 =99.99 %) against the nominal tyrosol concentrations.

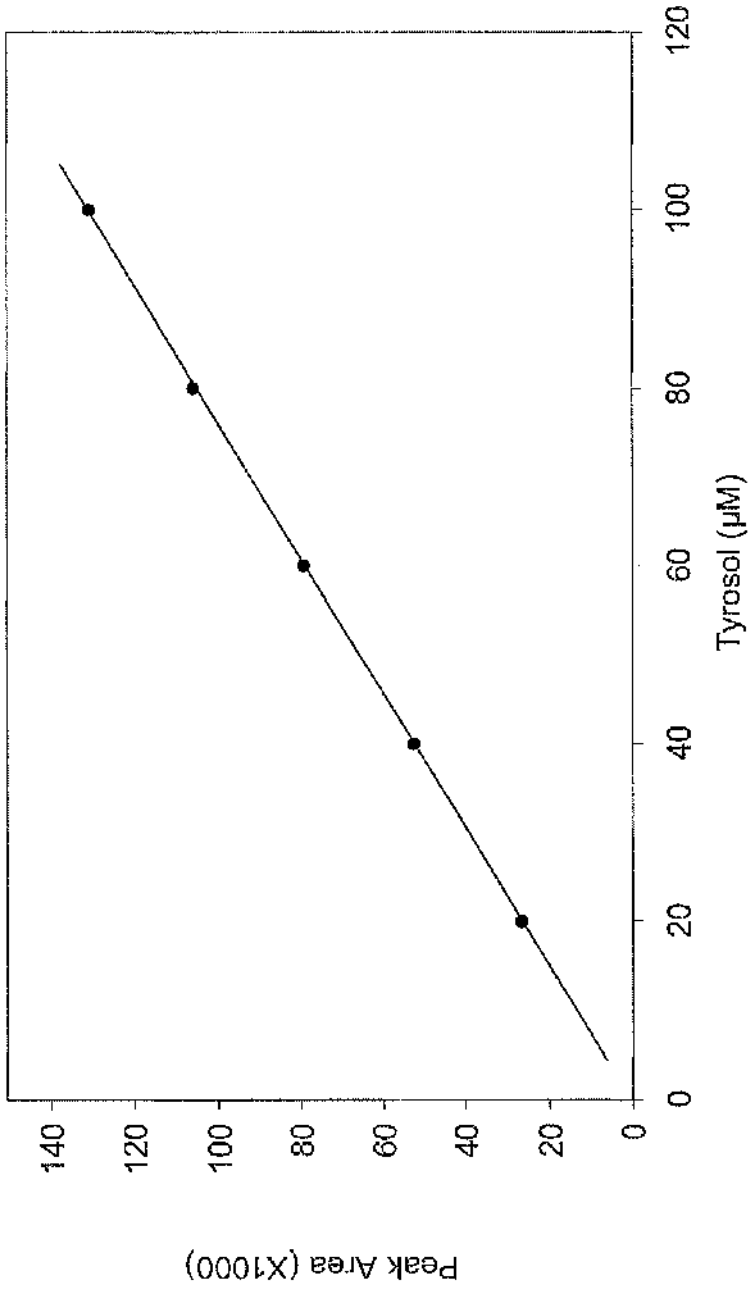

Peak Area (X1000)
0
20
40
60
80
100
120
140
Tyrosol (µM)
0
20
40
60
80
100
120

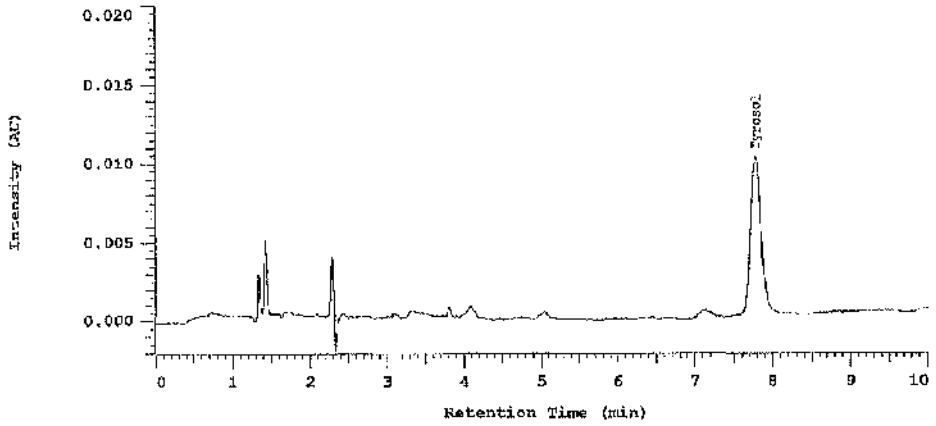
Figure 9

Typical HPLC chromatograms

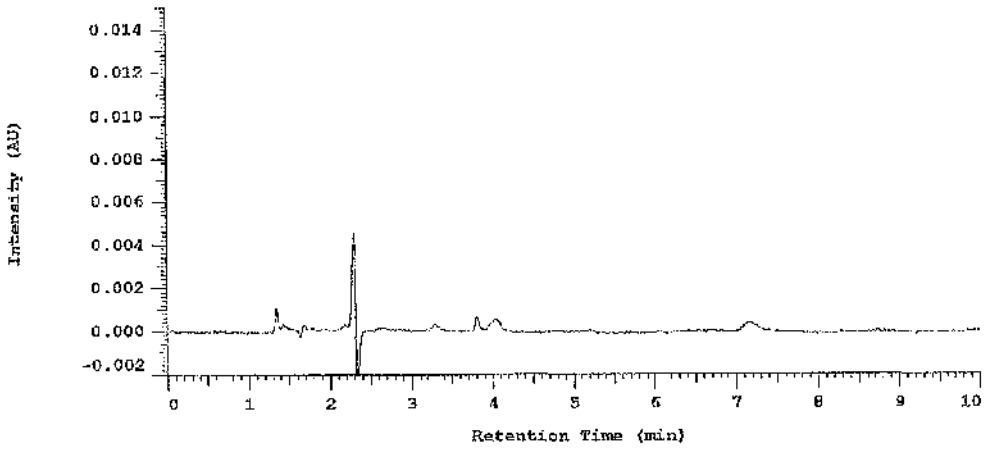
A) A supernatant extract containing 35 μM tyrosol (extracted by the developed SPE method). Chromatographic conditions: C-18 analytical column (Waters Spherisorb ODS2 5 μ, 4.6 x 250 mm). Mobile phase composition: t=0 to 10 min, 10% acetonitrile: 90% 1.0 mM H_2SO_4; t=10.1 to 20 min, 50% acetonitrile: 50% H_2O; t=20.1 to 30 min, 10% acetonitrile: 90% 1.0 mM H_2SO_4. The flow rate was 1.5 ml/min and the analysis was carried out at room temperature.

B) The growth medium (cleaned up by the developed SPE method) showing no interfering peaks at or around the tyrosol retention time (~ 7.7 min).

A


Intensity (AU)
0.020
0.015
0.010
0.005
0.000
Tyrosol
0 1 2 3 4 5 6 7 8 9 10
Retention Time (min)

B


Intensity (AU)
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
-0.002
0 1 2 3 4 5 6 7 8 9 10
Retention Time (min)

7.2 Effect of biofilm supernatant or farnesol on planktonic cell morphology

Initially, tissue culture flasks (75-cm^2 flat-bottom flasks) were used to prepare biofilm supernatant on a large scale to determine whether this culture supernatant could affect the morphology of planktonic cells grown in RPMI 1640 medium. A standardized cell suspension (1.0×10^6 cells/ml in RPMI 1640) was prepared as described previously (Section 5), and 30 ml of this suspension was added to 75-cm^2 tissue culture flasks. The cells were allowed to adhere for 1 h at 37 °C, the cell suspension was decanted to remove non-adherent cells and then fresh RPMI 1640 (40 ml) was added. Biofilms were allowed to form over 48h. The biofilm supernatant was then decanted, clarified by centrifugation at 3000 rpm, filter-sterilized through a 0.2 μm filter, and diluted 1:1 with a two-fold concentrate of RPMI 1640. Planktonic cells from an overnight culture were washed, resuspended and added to a flask containing 20 ml of this diluted supernatant to a density of 1.0×10^6 cells/ml. Flasks were incubated for 24h at 37 °C in an orbital shaker operating at 60 rpm. A negative control flask (RPMI 1640 only) and positive control flask (RPMI 1640 with 100 μM farnesol) were also included. Cell morphology was evaluated under a light microscope.

7.3 Effect of planktonic cell and biofilm supernatant on germ-tube formation

In this assay, germ-tube formation was used as an indicator to estimate the concentration of farnesol (and possibly other similar-quorum sensing molecules) produced during planktonic cell growth and biofilm formation. Cultures of *C. albicans* grown overnight in YNB-glucose medium were harvested and the cells were washed twice in 50 mM potassium phosphate buffer, pH 6.5. The cells were then resuspended at 1×10^7 cells/ml in 2.5 ml of a two-fold concentrate of the

same buffer containing 10 mM proline and 2.5 mM N-acetylglucosamine. This suspension was added to 2.5 ml of filter-sterilized supernatant from biofilms or planktonic cultures grown as described in Section 6.1.1. For a standard response curve, cells were resuspended at 1x 10^7 cells/ml in 5 ml of the same buffer containing 10 mM proline and 2.5 mM N-acetylglucosamine with farnesol at final concentrations ranging from 5 to 100 μM. Cell suspensions were incubated with gentle shaking at 37 °C for 3 h. At 0 min and 3 h, the suspensions were examined for the percentage of germ tubes present using a light microscope; 200 cells were counted each as described in Section 7.1.

7.4 Germ-tube formation in the presence of COX inhibitors

Cultures of *C. albicans* grown overnight in YNB-glucose medium were harvested and the cells were washed twice in 50 mM potassium phosphate buffer, pH 6.5. The cells were then resuspended at 10^7 cells / ml in the same buffer containing either 10 mM proline and /or 2.5 mM N-acetylglucosamine, or 5 % foetal bovine serum. Where indicated, at time zero, COX inhibitors were added to a final concentration of 100 μM, and PGE_2 was added to a final concentration of 1 nM, 100 nM or 1 μM. Cell suspensions were incubated with gentle shaking at 37 °C for 2 h. At 0 min and 2 h, the suspensions were examined for the percentage of germ tubes present using a light microscope; 200 cells were counted each time.

8. Scanning electron microscopy

8.1 Air-drying method

After biofilm formation, catheter disks were transferred to new 24-well plates and biofilms fixed with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer for 1h at room temperature. The biofilms were then rinsed twice for 5 min with 0.1 M phosphate buffer, treated with 1% (w/v) osmium tetroxide (Sigma) for 1h, washed three times with distilled water for 10 min, dehydrated in a series of ethanol solutions (30, 70, 90 and 100 %) for 10 min for each time, and finally treated with dried absolute ethanol for 10 min. The samples were left to air dry in a desiccator overnight, coated with gold using a Polaron coater and viewed under a Philips 500 scanning electron microscope or Joel 6400 scanning electron microscope.

8.2 Freeze-drying method

Biofilms were fixed with 2.5% (v/v) glutaradehyde in 0.1 M cacodylate buffer (pH 7.0) for 1h. Samples were then transferred to a new 24-well plate, washed gently three times in distilled water, and plunged into a liquid propane-isopentane mixture (2:1; v/v) at – 196 °C before freeze-drying under vacuum. A Polaron coater was used to coat the samples with gold before they were viewed under a Philips 500 scanning electron microscope or Joel 6400 scanning electron microscope.

9. Antifungal susceptibility testing by the NCCLS M27 method

9.1 Inoculum preparation

Five colonies of > 1 mm in diameter from 24-h culture plates (SDA) were resuspended in 5 ml saline (8.5 g/litre NaCl), and the resulting suspension was vortexed for 15 sec. The cell density was then adjusted to a reading of 0.5 at 530 nm using a spectrophotometer. This procedure yielded a yeast suspension of 1 x 10^6 to 5 x 10^6 cells/ml. A working suspension was made by a 1:100 dilution followed by 1:20 dilution of the stock suspension with RPMI 1640 medium buffered with 0.165 M MOPS (or another medium according to the experiment), which resulted in a yeast suspension of 0.5 x 10^3 to 2.5 x 10^3 cells/ml.

9.2 Microdilution method

Aspirin and/or amphotericin B solutions were prepared at twice the final concentration in RPMI 1640 medium buffered with 0.165 M MOPS (pH 7). The *C. albicans* inoculum was prepared as described previously (Section 9.1) except that the second dilution was 1:10 instead of 1:20. 100 µl of each agent or combined agent was placed in the wells of a microtitre plate. The final inoculum (100 µl) was added to each well of microtitre plate. This results in a 1:2 dilution of the agent or combined agent and also 1:2 of the inoculum. Control wells contained medium only, and inoculum with solvent but without any agent. The microtitre plates were incubated at 37 °C and observed for the presence or absence of visible growth. The plates were then read using a microplate reader at 490 nm.

9.3 Macrodilution method

This method was used to study the effect of COX inhibitors on the viability and optical density of planktonic cells of *C. albicans*. Stock solutions (100 mM; 10 µl) of aspirin, diclofenac, etodolac, salicylic acid and sodium salicylate were placed in 12 x 75 mm tubes. The control received 10 µl of dimethyl sulfoxide or ethanol without COX inhibitor. After the inoculum had been standardized (Section 10.1), 1 ml was added to each tube containing 10 µl of COX inhibitor and mixed. This resulted in a 1:100 dilution of each COX inhibitor to a final concentration of 1 mM.

10. Prostaglandin detection by ELISA

10.1 Preparation of samples

Prostaglandin production was determined in culture supernatants of planktonic cells and biofilms after concentrating the supernatant 10-fold by freeze drying. At the end of the incubation period, planktonic cells were centrifuged at 3000 rpm for 3 min. The supernatants were filtered through non-pyrogenic filters (pore size, 0.2 µm) and portions (5 ml) were freeze dried. For biofilms, after removal of disks with their adherent cells, the remaining medium from 12 wells was similarly filtered through non-pyrogenic filters (pore size, 0.2 µm) and portions (5 ml) were freeze dried. All freeze-dried samples were reconstituted by the addition of 0.5 ml of distilled water and analysed for prostaglandins using a prostaglandin screening enzyme immunoassay kit (Cayman Chemicals). All kit reagents (EIA buffer, wash buffer, prostaglandin acetylcholinesterase conjugate, prostaglandin antiserum and Ellman's reagent) were prepared according to the

manufacturer's instructions and only ultrapure water (Cayman Chemicals) was used. For standard curves, PGE_2 was diluted in a ten-fold concentrate of yeast nitrogen base medium.

10.2 ELISA plate assay

Controls, standards and samples were assayed in special 96-well plates pre-coated with mouse anit- rabbit IgG, included with the kit. For every experiment, each plate or set of strips contained two wells of blanks, two non-specific binding wells, two maximum binding wells, one total activity well and an eight-point standard curve run in duplicate. Each sample was assayed at a minimum of two dilutions (Table 2). To check for possible interference by COX inhibitors, additional controls were included that contained a 10-fold concentrate of medium plus inhibitors. Each plate or set of strips was covered with plastic film supplied with the kit, and incubated for 18 h at 23 °C.

10.3 ELISA plate development and reading

To develop the plate, wells were emptied and rinsed five times with wash buffer. Ellman's reagent (200 µl) was added to each well and 5 µl of tracer was added to the total activity well. The wells were covered with plastic film, and the plate was placed in an orbital shaker, and allowed to develop in the dark. The plate was read in a microtitre plate reader at a wavelength of 405 nm after 60-90 min when the absorbance of the maximum binding wells was in the range of 0.3-0.8.

Table 2. ELISA plate pipetting summary

Well	EIA Buffer	Standard/Sample	Tracer	Antibody
Blank	---	---	---	---
Total activity	---	---	5 µl *	---
Non specific-binding	100 µl	---	50 µl	---
Maximum binding	50 µl	---	50 µl	50 µl
Standard/Sample	---	50 µl	50 µl	50 µl

* Added at development of the plate.

11. Determination of cell dry weight

Growth of planktonic cells and biofilms was measured by determination of cell dry weight. At the end of the incubation period, portions of planktonic cell cultures (3 ml) were collected on preweighed cellulose nitrate filters (0.45 μm pore size; 25 mm diameter) and given three washes with water (5 ml). The filters were dried to constant weight at 37 °C, and the dry weights of cells per filter were calculated. Dry weights were determined in triplicate. After biofilm formation, 12 disks with their adherent cells were transferred to 12 ml of 0.15 M phosphate-buffered saline, pH 7.2, and vortexed vigorously. Portions (3 ml) of the resulting cell suspensions were then collected on preweighed cellulose nitrate filters and processed as described above.

12. Statistical analysis of data

Standard errors of the mean are quotes for all quantitative assays of biofilm formation. Student's t-test was used to detect significant differences between mean values and the results are presented as P values. All statistical tests were carried out using Microsoft Excel 2003.

RESULTS

1. Preliminary studies on biofilms and germ-tube development in *C. albicans*

1.1 Quantitative measurement of biofilm formation using the XTT assay

Initial experiments on biofilm formation were carried out using an XTT assay procedure in which the tetrazolium salt was dissolved in YNB medium. A yellow colour was produced and the A_{492} readings were quite low, ranging from 0.100 to 0.300, with average value of 0.230 ± 0.082 (Fig. 10). When XTT was dissolved in PBS containing 1% glucose instead of YNB medium, a dark orange color was produced in the assay and A_{492} readings ranged from 1.220 to 1.600, with an average reading of 1.450 ± 0.066 (Fig. 10). For this reason, in all subsequent experiments XTT was dissolved in PBS containing glucose to enhance the metabolic activity of biofilm cells and so produce a higher level of XTT reduction.

1.2 Determination of optimal glucose concentration for the XTT assay

To determine the optimal concentration of glucose for the XTT reduction assay, PBS solutions containing different glucose concentrations (0, 0.2, 0.4, 0.6, 0.8 and 1%) were tested (Fig. 11). There was a steady increase in A_{492} reading with increasing glucose concentration, and a maximal A_{492} value (1.845) was reached at 1% glucose (Fig. 11).

Figure 10

Quantitative measurement of biofilm formation by *C. albicans* GDH 2346 using the XTT assay

Biofilms were grown on PVC catheter disks for 48h at 37°C. Quantitative measurements of biofilm metabolic activity were made using the XTT assay with XTT dissolved in YNB or PBS +1% glucose. The results are means ± SEM from two independent experiments done in triplicate.

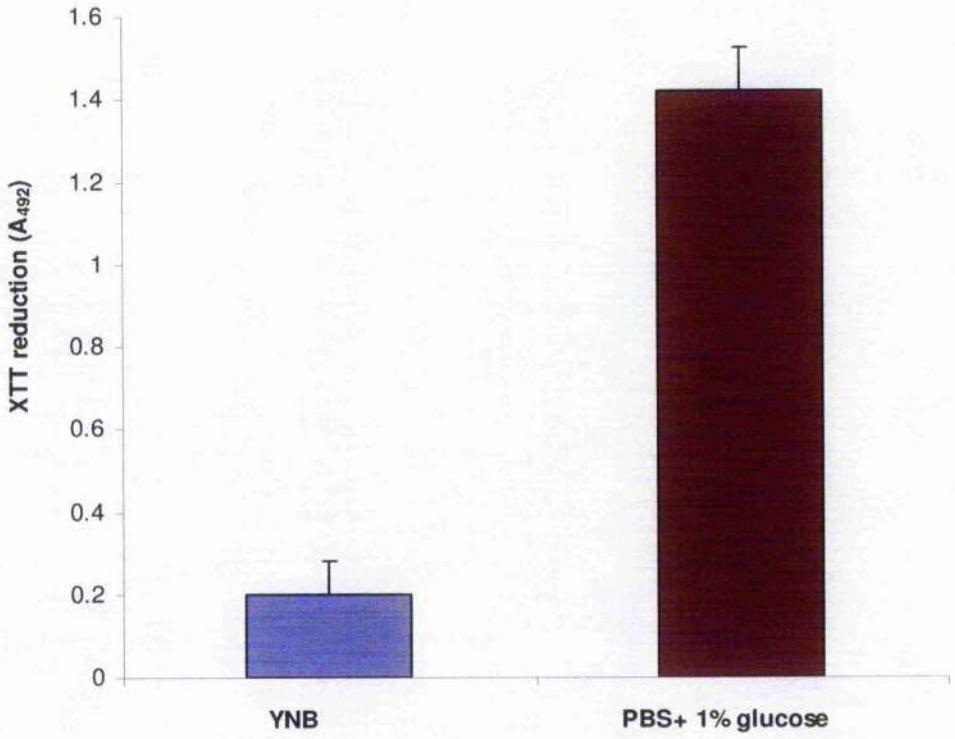
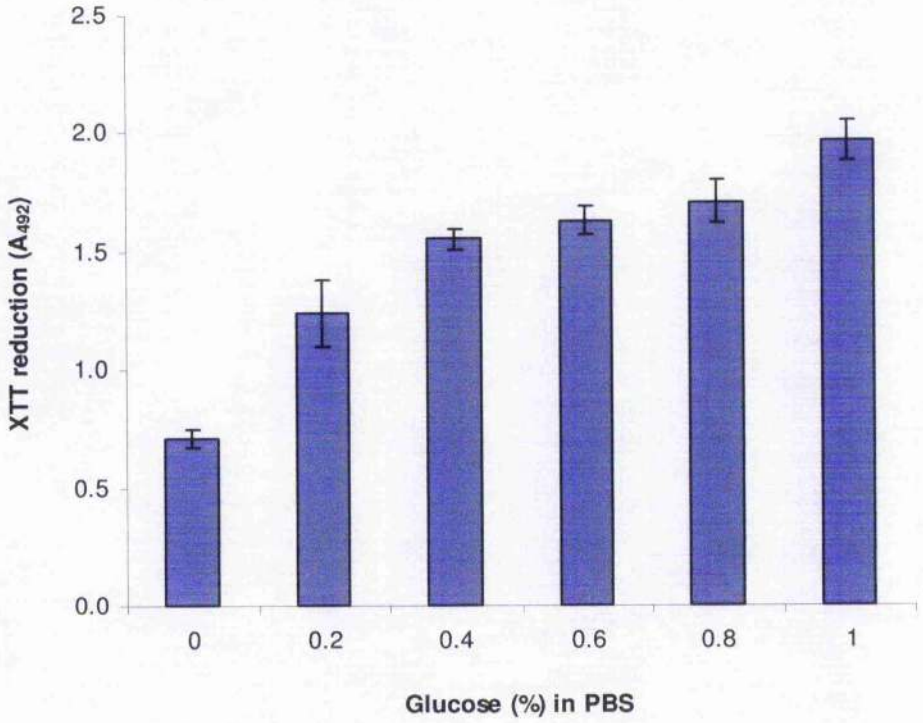

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
XTT reduction (A_{492})
YNB
PBS+ 1% glucose

Figure 11

Effect of different glucose concentrations in the XTT assay for measurement of biofilm formation by *C. albicans* GDH 2346

Biofilms were grown on PVC catheter disks for 48h at 37°C. Quantitative measurements of biofilm metabolic activity were made using the XTT assay with XTT dissolved in PBS supplemented with different glucose concentrations. The results are means ± SEM from two independent experiments done in triplicate.


2.5
2.0
1.5
1.0
0.5
0.0
XTT reduction (A_{492})
0
0.2
0.4
0.6
0.8
1
Glucose (%) in PBS

1.3 Standard curve of planktonic cell count against OD $_{520}$ reading

A haemocytometer slide was used to count planktonic cells of *C. albicans* in order to standardize cell suspensions. In this experiment, *C. albicans* cell numbers were related to the optical density at 520nm (Fig. 12). There was a linear relationship between cell number (log $_{10}$ cells/ml) and OD_{520} for optical density readings between 0.600 and 1.600 (Fig. 12).

1.4 Biofilm formation by *C. albicans* strains obtained from the University of Nebraska.

The *C. albicans* strains used in these experiments were provided by Jacob M. Hornby (School of Biological Sciences, University of Nebraska). They include *C. albicans* CAI-4 (wild-type strain) and six mutants with altered colony morphology and farnesol-mediated quorum sensing. Biofilm formation by these strains was evaluated by the catheter disk method; biofilms were allowed to grow for 48h and biofilm metabolic activity was measured by XTT reduction. Biofilm formation (XTT reduction) by the mutants was calculated as a percentage of that of the wild type strain CAI-4 (Fig. 13). The results showed no significant difference in biofilm formation between the wild type and mutants. Biofilm formation by the mutants was 88 to 108 % of that of *C. albicans* CAI-4 (Fig. 13).

1.5 Biofilm formation by *C. albicans* strains obtained from Columbia University, New York

Biofilm formation by *C. albicans* mutants defective in the *MDS3* gene which is necessary for alkaline pH-induced responses (Davis *et al.*, 2002), or the *RIM101* pathway which is required for *in vivo* pathogenesis (Davis *et al.*, 2000)

were investigated. The strains were: *C. albicans* DAY185 (wild type), *C. albicans* VIC25 (*mds3/mds3*), DAY25 (*rim101/rim101*) and VIC28 (*rim101/rim101 mds3/mds3*). The results (Fig. 14) demonstrate that biofilm formation by *C. albicans* VIC25 and VIC28 was significantly less than that of the wild type by 27 and 11 %, respectively ($P< 0.01$ and $P <0.05$). However, biofilm formation by *C. albicans* DAY25 was 10% greater than that of the wild type ($P<0.05$).

1.6 Biofilm formation by *C. albicans* SC 5314 and its morphological mutants

Germ-tube formation is important in the expression of *C. albicans* pathogenicity. In these experiments, biofilm formation by morphological mutants blocked in one of two signalling pathways (*cph1/cph1* or *efg1/efg1*) or in both pathways (*cph1/cph1 efg1/efg1*), was investigated. The results (Fig 15), showed that biofilm formation by the morphological mutants was similar to that of the wild-type strain, as determined by the XTT assay. This indicates that the blockages in these signalling pathways did not affect biofilm formation by any of the mutants. The structure and cell morphology of these biofilms is described later (Section 2.12).

Figure 12

Standard curve of planktonic cell count against O.D $_{520}$ reading for *C. albicans* **GDH 2346**

The results are the means of two independent experiments done in duplicate. *C. albicans* cells were counted in a haemocytometer.

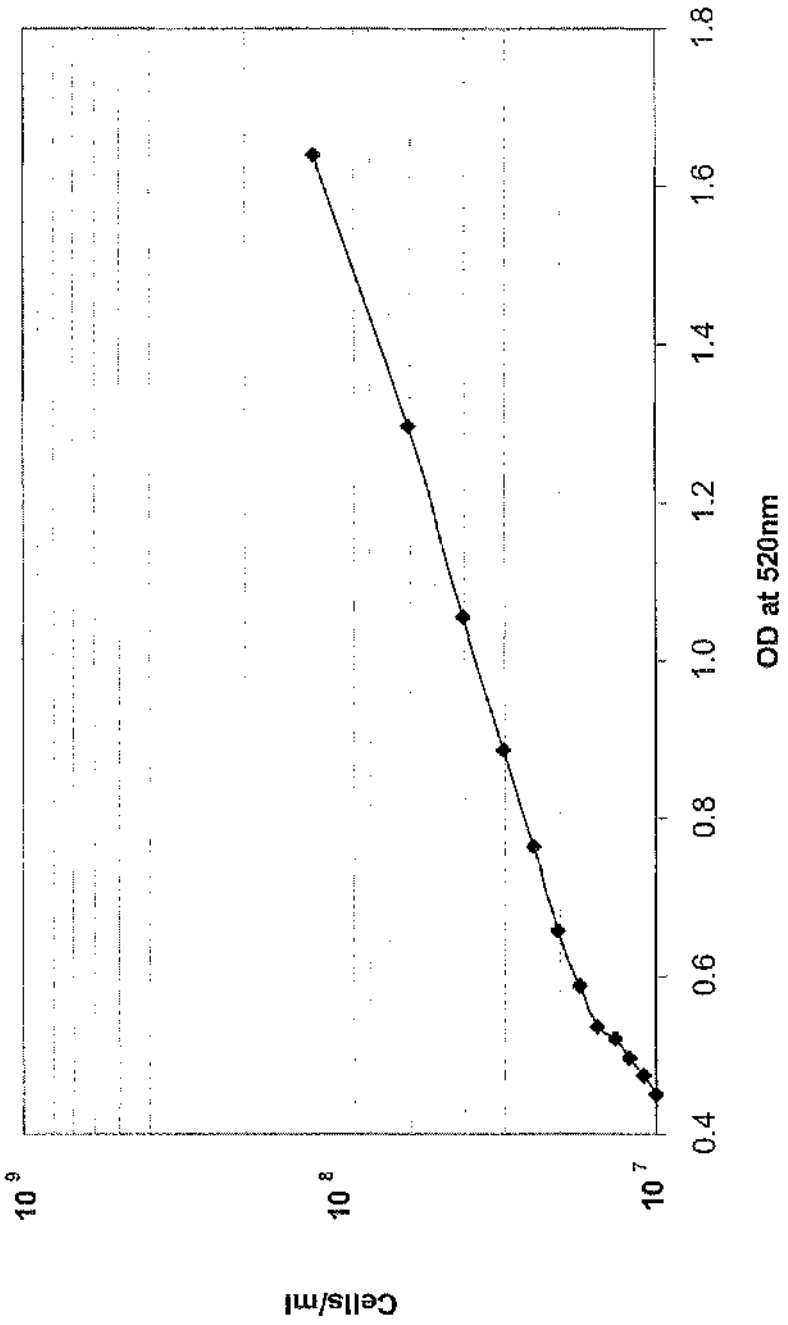

Cells/ml
10^9
10^8
10^7
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
OD at 520nm

Figure 13

Biofilm formation by *C. albicans* strains obtained from the University of Nebraska

Biofilm formation (as measured by XTT reduction) is expressed as a percentage of that of the wild-type strain *C. albicans* CAI-4. The results are means ± SEM of four independent experiments carried out with at least three replicates. The mean (± SEM) A_{492} value for *C. albicans* CAI-4 was 2.685 ± 0.142.

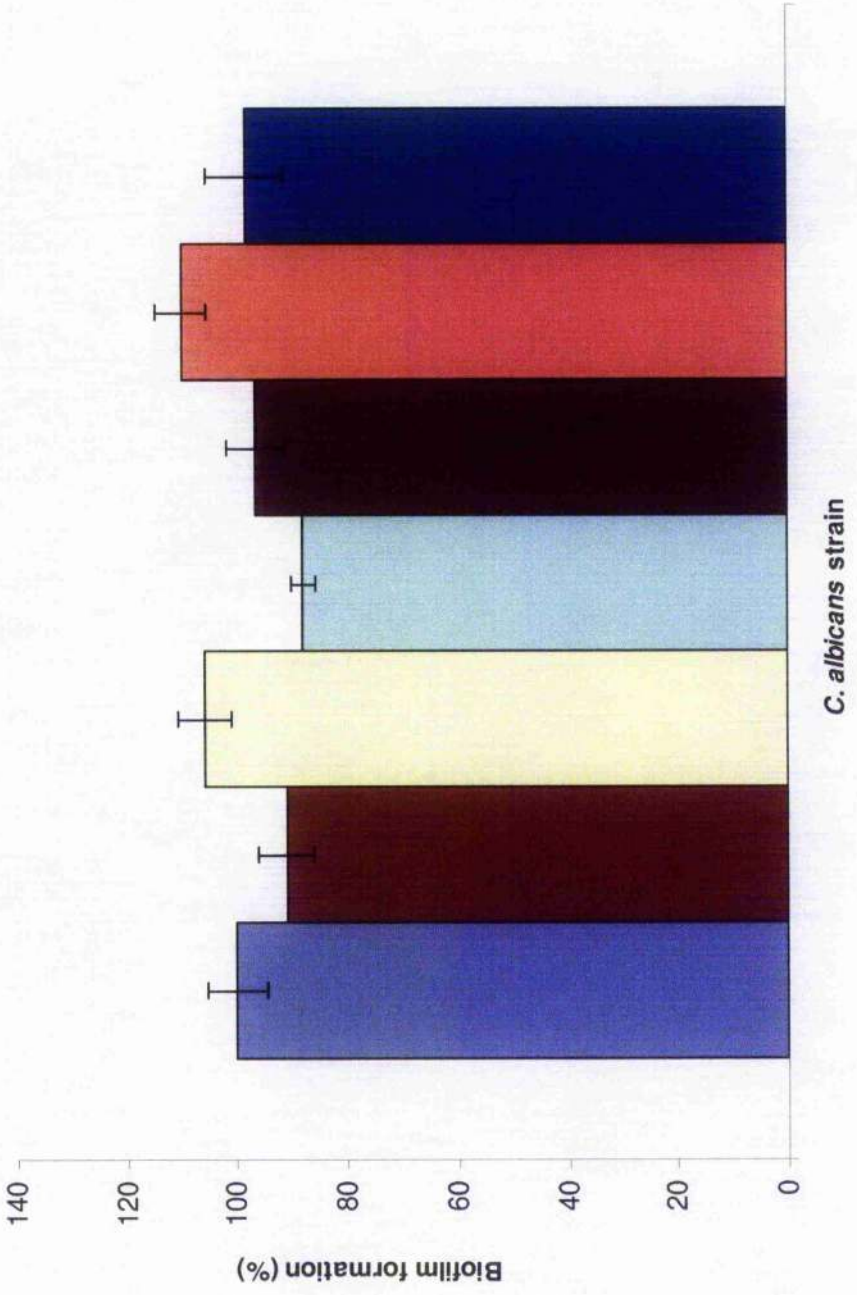

Biofilm formation (%)
0
20
40
60
80
100
120
140
C. albicans strain

Figure 14

Biofilm formation by *C. albicans* strains obtained from the Columbia University, New York

Biofilm formation (as measured by XTT reduction) is expressed as a percentage of that of the wild-type strain *C. albicans* DAY185. The results are means ± SEM of three independent experiments carried out in triplicate. The mean (± SEM) A_{492} value for *C. albicans* DAY185 was 2.732± 0.082.

- DAY 185
- VIC 25
- DAY 25
- VIC 28

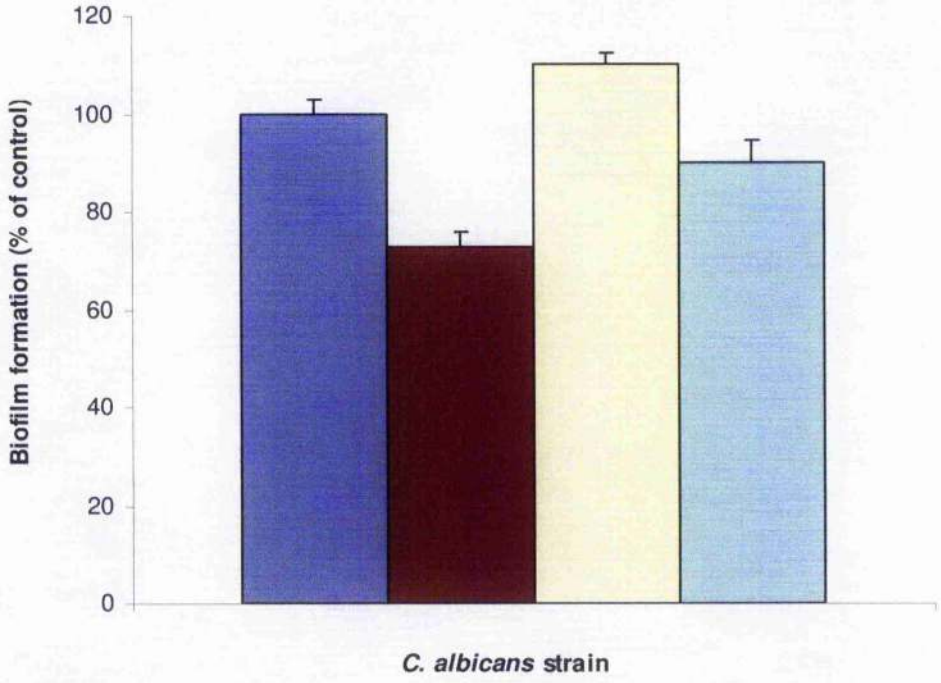

120
100
80
60
40
20
0
Biofilm formation (% of control)
C. albicans strain

Figure 15

Biofilm formation by *C. albicans* SC 5314 and its morphological mutants

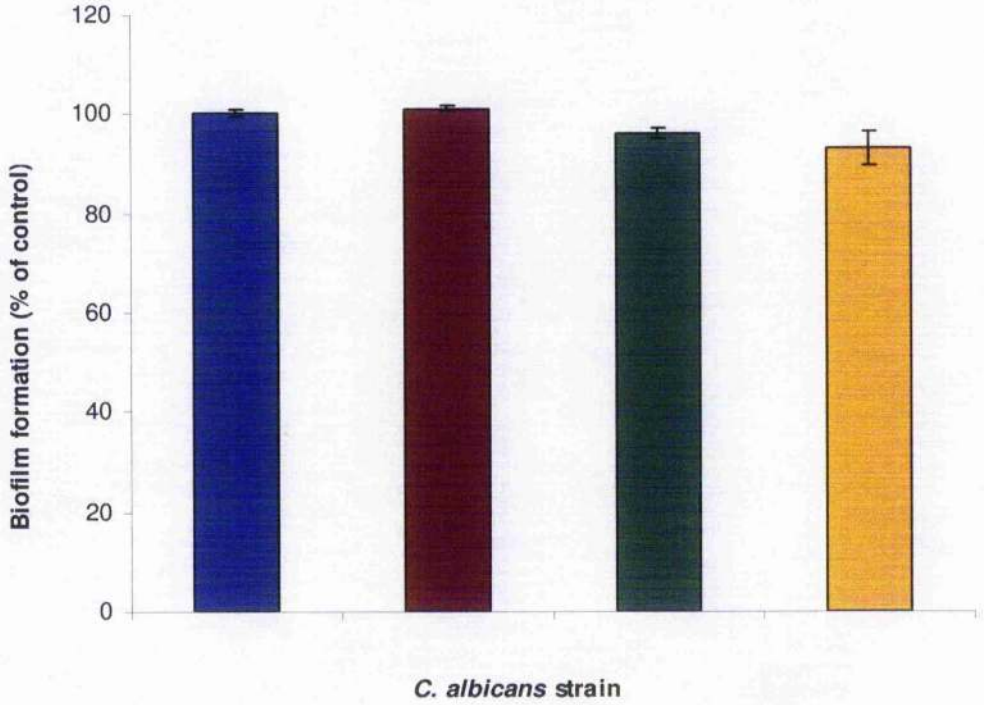
Biofilm formation (as measured by XTT reduction) is expressed as a percentage of that of the wild-type strain *C. albicans* SC5314. The results are means ± SEM of two independent experiments done in triplicate. The mean (± SEM) A_{492} value for *C. albicans* SC5314 was 2.402± 0.321.


C. albicans SC5314


C. albicans JKC 19 (*cph1/cph1*)


C. albicana HLC 52 (*efg1/efg1*)


C. albicans HLC 54 (*cph1/cph1 efg1/efg1*)


120
100
80
60
40
20
0
Biofilm formation (% of control)
C. albicans strain

1.7 Germ-tube formation with different inducers

The ability of *C. albicans* to form germ tubes varies according to the growth conditions and the germ-tube inducers used. In preliminary experiments on germ-tube formation, 5% serum caused problems in counting because the cells clumped together. For this reason, two other inducers were used: 10 mM proline (Pro) and 2.5mM N-acetylglucosamine (GlcNAc). These inducers were used individually or as a mixture. Germ-tube formation was evaluated with and without gentle shaking to establish the optimal condition for counting the cells. The results show that both inducers activate budding yeast cells to form germ tubes (Fig. 16). Maximal germ-tube formation was achieved using a mixture of proline and N-acetylglucosamine with gentle shaking; figures of 55 % and 86% germ-tube formation were obtained at 2h and 5h, respectively. In contrast, at the same time periods only 14% and 28% of cells formed germ tubes in the presence of proline alone without shaking. Therefore treatment with a mixture of 10 mM proline and 2.5 mM N-acetylglucosamine, with gentle shaking, was used as the standard method for germ-tube formation.

1.8 Germ-tube formation in the presence of farnesol

Farnesol is a quorum-sensing molecule which inhibits germ-tube formation (Hornby *et al.*, 2001). Before evaluating the effect of farnesol on biofilm formation, it was of interest to study its effect on *C. albicans* germ-tube formation. All of the *C. albicans* strains used here were incubated in the presence of the proline-N-acetylglucosamine inducer. The results show that most strains were able to form germ tubes and the majority gave over 40% germ-tube

formation within 3h (Fig. 17). Two strains defective in signalling pathways (HLC 52 and HLC 54) were the exception and failed to produce any germ tubes. With all the other strains farnesol (100 μM) completely blocked germ-tube formation (data not shown).

1.9 Effect of propranolol and 1-oleoyl-2-acetyl-sn-glycerol (OAG) on germ-tube formation by *C. albicans* GDH 2346

Propranolol (Prop) was reported in previous studies to inhibit germ-tube formation in *C. albicans* by binding with phosphatidic acid which is involved in switching on filamentous growth (Baker *et al.*, 2002). In *S. cerevisiae* growth is inhibited in medium containing farnesol; this inhibition can be abolished by coaddition of diacylglycerol analogues such as OAG (Machida et al., 1999). Consequently, it was of interest to investigate the effect of these compounds on germ-tube formation by some of the *C. albicans* strains used in this study. Initially *C. albicans* GDH 2346 was selected as a model strain in these experiments because it has been well studied for its ability to form germ tubes and biofilms. The results (Fig. 18) show that 100 μM farnesol completely inhibited germ-tube formation, while 100 μM and 1 mM propranolol inhibited germ-tube formation by 31 % and 88 %, respectively ($P<0.05$ and $P<0.01$). In further experiments, similar results were found for *C. albicans* SC5314 and JKC19. Farnesol at 0.1 mM and 1 mM blocked germ-tube formation completely while 1 mM propranolol inhibited the process by more than 78% ($P< 0.001$; Table 3).The effect of OAG was studied only with *C. albicans* GDH 2346. This compound on its own did not increase germ-tube formation by *C. albicans* GDH 2346 as compared with the control (Fig. 18). However, OAG decreased the ability of farnesol to block germ-tube

formation; 38% of the cells were able to form germ tubes in the present of both 100 μM farnesol and 1 mM OAG (Fig. 18).

Figure 16

Germ-tube formation by *C. albicans* GDH 2346 in the presence of different inducers

Yeast cells were suspended in 50 mM potassium phosphate buffer (pH 6.5), containing 10 mM proline (Pro) and / or 2.5 mM N-acetylglucosamine (GlcNAc) and incubated for 5h at 37°C with and without gentle shaking. Results are means ± SEM of two determinations, with 200 cells counted.

◆ Pro

■ Pro with gentle shaking

▲ Pro + GlcNAc

□ Pro + GlcNAc with gentle shaking

△ GlcNAc

◇ GlcNAc with gentle shaking

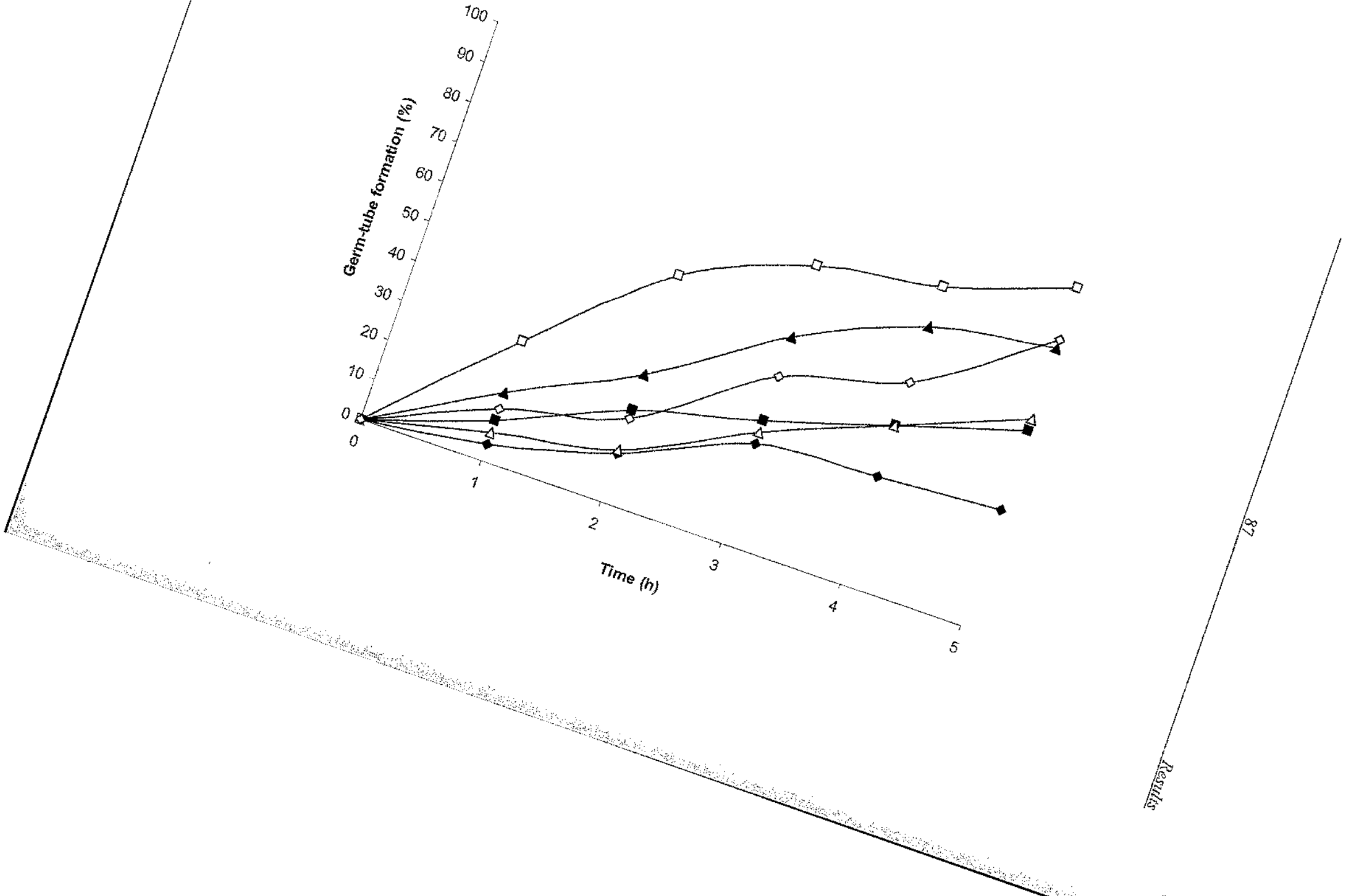

Germ-tube formation (%)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
Time (h)
Results
87

Figure 17

Germ-tube by formation of different *C. albicans* strains

Yeast cells were suspended in 50 mM potassium phosphate buffer (pH 6.5), containing 10 mM proline (Pro) and 2.5 mM N-acetylglucosamine (GlcNAc), and incubated for 5h at 37°C with gentle shaking. Results are means ± SEM of two determinations, with 200 cells counted.

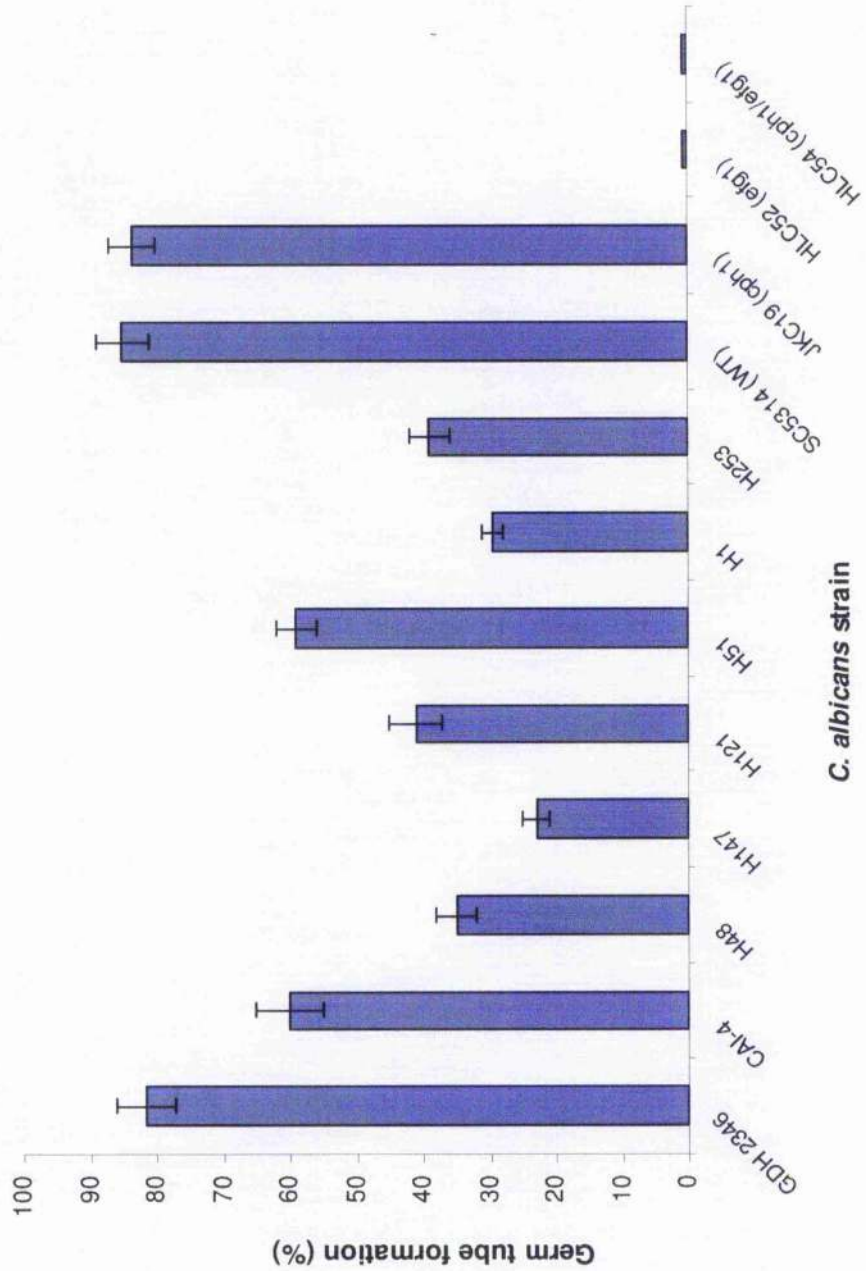

Germ tube formation (%)
0
10
20
30
40
50
60
70
80
90
100
GDH 2346
CAI-4
H48
H147
H121
H51
H1
H253
SC5314 (WT)
JKC19 (cph1)
HLC52 (efg1)
HLC54 (cph1efg1)
C. albicans strain

Figure 18

Effects of farnesol, propranolol and OAG on germ-tube formation by *C. albicans* GDH 2346

Germ-tube formation is expressed as a percentage of that of control cells incubated in the absence of any agents. Results are means ± SEM of triplicate determinations. The mean (± SEM) control value was 170 ± 10 germ tube-forming cells / 200 cells counted.

Far = Farnesol

Prop = Propranolol

OAG = 1-oleoyl-2-acetyl-sn-glycerol

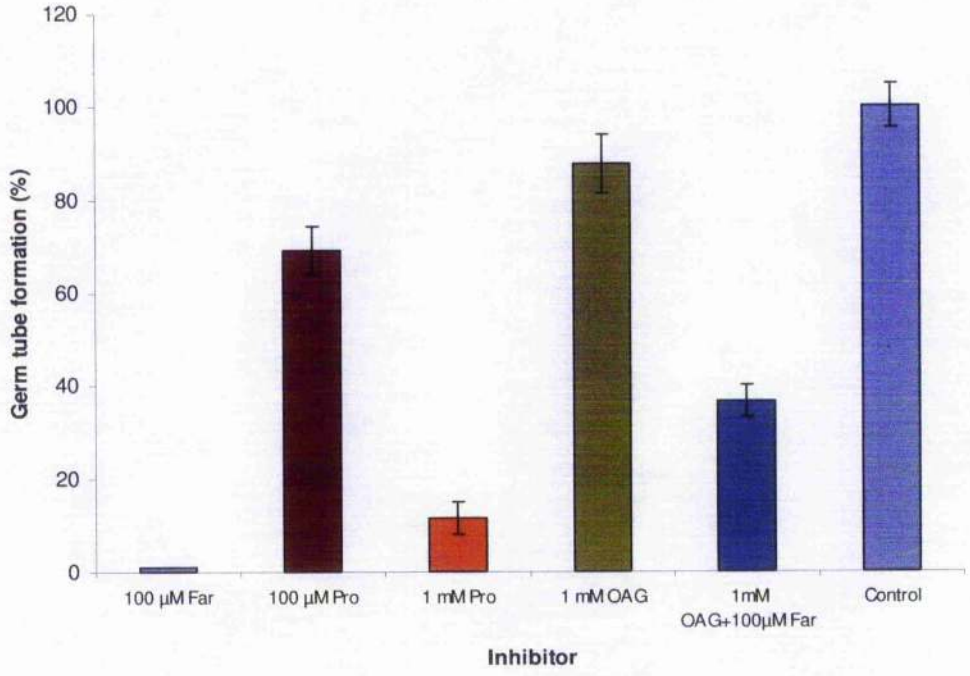

120
100
80
60
40
20
0
Germ tube formation (%)
100 µM Far
100 µM Pro
1 mM Pro
1 mM OAG
1mM OAG+100µM Far
Control
Inhibitor

Table 3. Germ-tube formation by *C. albicans* SC5314 and its morphological mutants

C. albicans strain	Germ tube (%) in the presence of :			
	Control	100 µM Farnesol	1 mM Farnesol	1 mM Propranolol
SC5314	100	0	0	22 ± 3 [a]
JKC 19	100	0	0	21 ± 3 [a]
HLC 52	0	0	0	0
HLC 54	0	0	0	0

Germ-tube formation is expressed as a percentage of that of control cells incubated in the absence of farnesol or propranolol. Results are means ± SEM of two independent experiments done in duplicate. The mean (± SEM) control value for *C. albicans* SC5314 was 170 ± 8 and for *C. albicans* JKC 19 was 168 ± 6 germ tube-forming cells /200 cells counted.

[a] Value significantly different at $P < 0.001$ from that of control.

2. Role of farnesol as a quorum sensing molecule in *C. albicans* biofilms

Farnesol is known to inhibit both the dimorphic transition and biofilm formation in *C. albicans* (Hornby *et al.*, 2001; Oh *et al.*, 2001; Ramage *et al.*, 2002a). Recently, tyrosol was identified as another quorum sensing molecule which promotes hyphal formation (Chen *et al.*, 2004). However, the mechanisms by which the two molecules interact to regulate *C. albicans* biofilm development are unknown. In this section the effect of farnesol on *C. albicans* biofilms was investigated.

2.1 Biofilm formation by *C. albicans* GDH 2346 in the presence of farnesol

Farnesol at three different concentrations (50 μM, 100 μM and 1M) was added at different stages of biofilm development in YNB medium. Only the early stages of biofilm formation were sensitive to the effect of farnesol (Fig. 19), i.e. when farnesol was added during the adhesion period and again at time zero, so that it was present throughout biofilm formation. For example, 50 and 100 μM farnesol inhibited biofilm formation by 14 and 19%, respectively ($P<0.05$), when added at this stage. Maximal inhibition of 33% ($P<0.001$) was observed with 1 mM farnesol, again when added during the adhesion period and maintained throughout biofilm formation (Fig. 19).

Figure 19

Effect of farnesol concentration on biofilm formation of *C. albicans* GDH 2346 grown in YNB medium

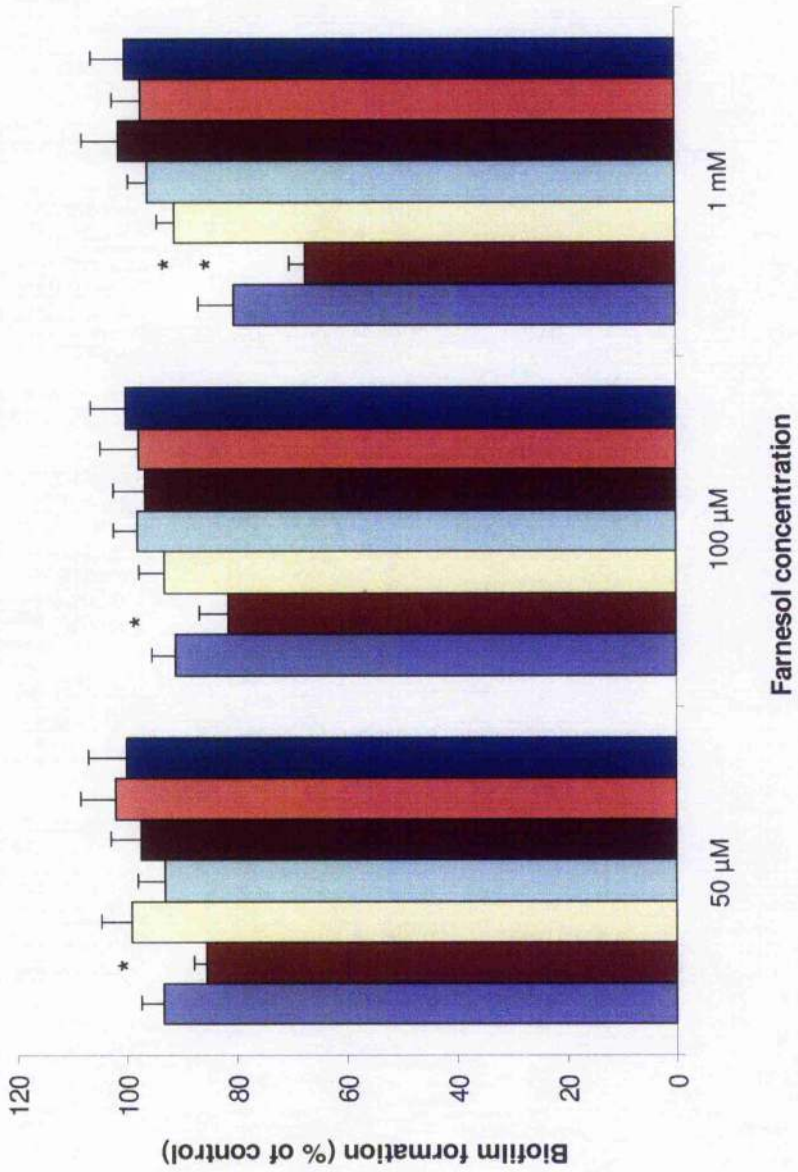
Different farnesol concentrations (50 μM, 100 μM, and 1 mM) were added at different stages of biofilm development. Biofilm formation (XTT reduction) is expressed as a percentage of that of control biofilms incubated in the absence of farnesol. Results are means ± SEM from at least two independent experiments carried out in triplicate. Mean (± SEM) control values (A_{492}) ranged from 1.999 ± 0.399 to 2.311± 0.142.

Farnesol was added at:

- Adhesion
- Adhesion and time zero
- Time zero
- 2h of biofilm formation
- 4h of biofilm formation
- 24h of biofilm formation
- Control biofilm (no farnesol added)

* $P < 0.05$ compared with control

* * $P < 0.001$ compared with control


Biofilm formation (% of control)
120
100
80
60
40
20
0
*
*
*
*
50 µM
100 µM
1 mM
Farnesol concentration

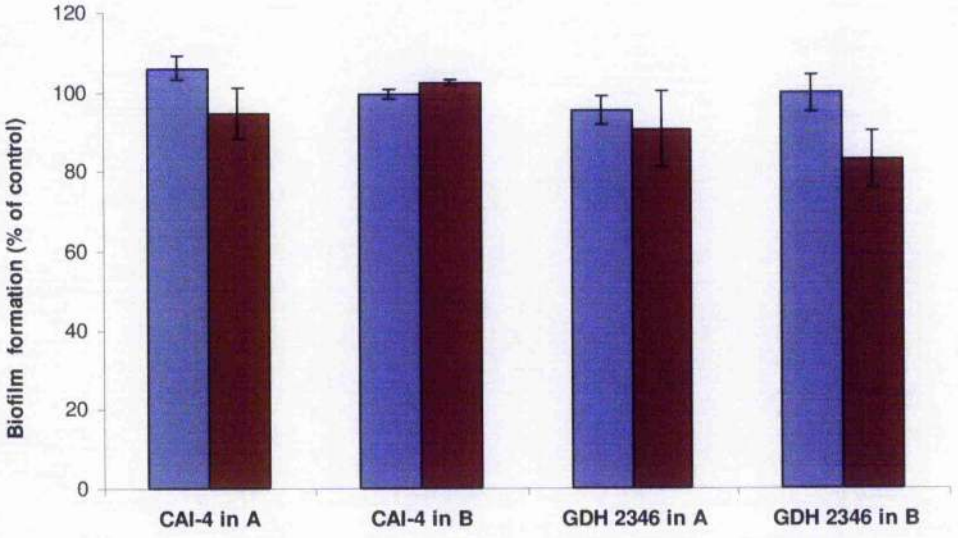
2.2 Effect of 1 mM farnesol on biofilm formation by *C. albicans* GDH2346 and *C. albicans* CAI-4 grown on Hornby medium or RPMI 1640

The previous experiment showed that 1 mM farnesol inhibited biofilm formation by *C. albicans* GDH 2346 by up to 33% when the organism was grown in YNB. In order to determine whether growth medium composition could affect farnesol activity against *C. albicans* biofilms, YNB medium was replaced by two different media which enhance germ-tube formation: Hornby medium and RPMI 1640. Farnesol was added for the 1h adhesion period only or for the adhesion period and throughout biofilm formation. The results showed that farnesol did not significantly affect biofilm formation by *C. albicans* CAI-4 when added at either stage in either growth medium (Fig. 20). Similar results were found for *C. albicans* GDH 2346 grown in Hornby medium. In contrast, 1 mM farnesol inhibited biofilms of strain GDH 2346 grown in RPMI 1640 medium by 17% ($P < 0.01$) when added for the adhesion period and maintained throughout biofilm formation (Fig. 20).

Figure 20

Effect of farnesol on *C. albicans* biofilms grown in Hornby medium or RPMI 1640 medium

Biofilm formation by *C. albicans* GDH 2346 and *C. albicans* CAI-4 (as measured by XTT reduction) is expressed as a percentage of that of control biofilms incubated in the absence of 1 mM farnesol. Results are means ± SEM from two independent experiments with a total of six replicates. Mean (± SEM) control values (A_{492}) in Hornby medium were 0.964 ± 0.105 and 2.577 ± 0.182 for *C. albicans* GDH 2346 and *C. albicans* CAI-4 respectively, while in RPMI 1640 they were 2.927 ± 0.262 and 3.226 ± 0.075. Farnesol (1 mM) was added at the start of the adhesion period (Ad, ▨) or at adhesion and again at time zero of biofilm formation (Ad + 0h, ■). A, Hornby medium; B, RPMI 1640 medium.


Biofilm formation (% of control)
120
100
80
60
40
20
0
CAI-4 in A
CAI-4 in B
GDH 2346 in A
GDH 2346 in B

2.3 Biofilm formation by *C. albicans* GDH 2346 in the presence of farnesol and 1-oleoyl-2-acetyl-sn-glycerol (OAG)

Previous results showed that OAG partially prevented farnesol from blocking germ-tube formation. In this experiment, 1 mM OAG was added together with farnesol during biofilm formation by *C. albicans* GDH 2346 to determine whether OAG could affect the inhibitory activity of farnesol on biofilm formation. Initially, 1 mM farnesol and 1 mM OAG were added individually or together at adhesion and time zero for biofilms grown in YNB. The results (Fig. 21) showed that 1 mM farnesol inhibited biofilm formation by 24 % ($P<0.01$). However, the inhibitory effect of farnesol was abolished when biofilms were grown in the presence of both 1 mM farnesol and 1 mM OAG (Fig. 21).

2.4 Scanning electron microscopy of the effects of farnesol, propranolol and OAG on *C. albicans* GDH 2346 biofilms grown in YNB, RPMI 1640 and Hornby medium

It is known that *C. albicans* biofilms grown on catheter disks in YNB medium normally contain two layers: a thin, basal region of densely packed yeast cells and an overlying, mostly hyphal layer (Baillie and Douglas, 1999b). Therefore in this investigation the effects of farnesol, propranolol and OAG on the structure of *C. albicans* GDH 2346 biofilms grown in YNB, RPMI 1640 and Hornby medium were studied. As can be seen from the scanning electron micrographs in Fig. 22, control biofilms grown in YNB medium consisted of a mixture of yeasts and hyphae (A-1), whereas control biofilms grown in Hornby medium and RPMI 1640 consisted mainly of hyphae (A-2 and A-3). Biofilms treated with 1 mM farnesol or 1 mM propranolol in all growth media consisted of

yeast cells only (Fig. 22, B-1,2,3 and C-1,2,3). However, OAG did not change the structure of biofilms grown in the three media; they retained the morphology of control biofilms (Fig. 22, D-1, 2, 3). When farnesol was added together with OAG mainly yeast-only biofilms were formed, apart from biofilms grown in RPMI 1640 which contained a small number of hyphae (Fig. 22, E-1, 2 and 3). Similar results were obtained with biofilms grown in the presence of 1 mM OAG and propranolol, although biofilms grown in Hornby medium contained a few hyphae (Fig. 22, F- 1, 2 and 3).

Figure 21

Effect of farnesol and /or OAG on biofilm formation by *C. albicans* GDH 2346

Biofilm formation by *C. albicans* GDH 2346 (as measured by XTT reduction) is expressed as a percentage of that of control biofilms incubated in the absence of 1 mM farnesol or 1 mM OAG. Farnesol and OAG were added at adhesion and time zero of biofilm formation. Results are means ± SEM from three independent determinations. Mean (± SEM) control value (A_{492}) was 1.971 ± 0.095.

Additions:

- 1 mM OAG
- 1 mM OAG+1 mM farnesol
- 1 mM farnesol
- Control (addition of equal volume of methanol)

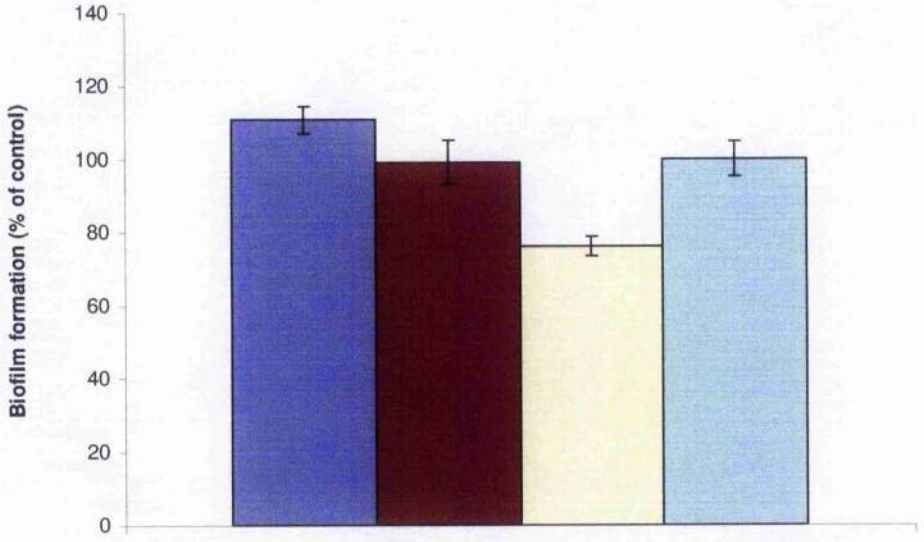
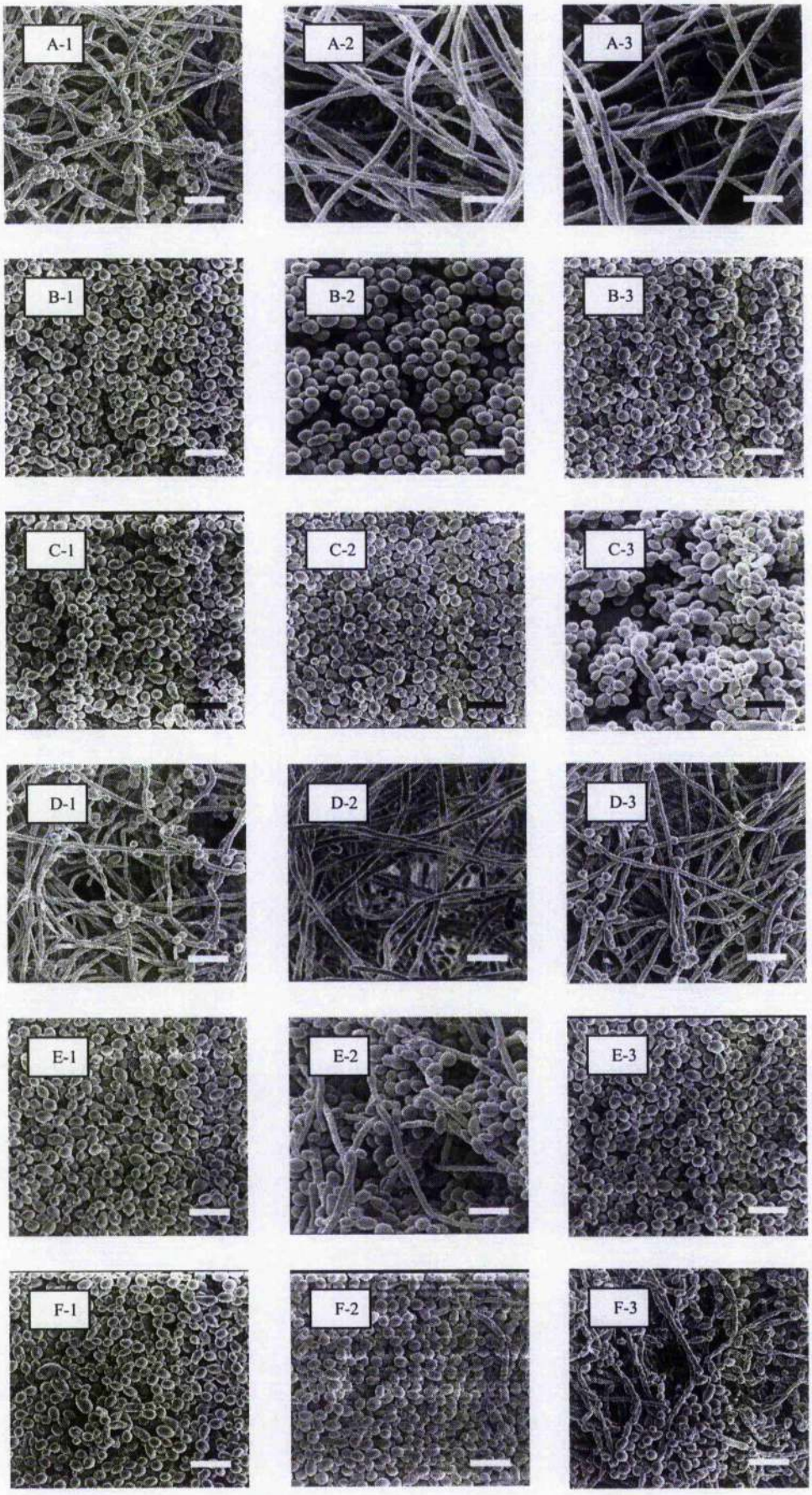

Biofilm formation (% of control)
140
120
100
80
60
40
20
0

Figure 22

Scanning electron micrographs of the effects of farnesol, propranolol and OAG on biofilms of *C. albicans* GDH 2346 grown in three different media

C. albicans GDH 2346 biofilms were grown on PVC disks for 48h in (1) YNB medium; (2) RPMI 1640; (3) Hornby medium. A, control biofilms; B, with 1 mM farnesol; C, with 1 mM propranolol; D, with 1mM OAG; E, with 1 mM farnesol and OAG; (F) with 1 mM propranolol and 1 mM OAG. All agents were added at adhesion and time zero of biofilm formation. Bar, 8 μm.


A-1
A-2
A-3
B-1
B-2
B-3
C-1
C-2
C-3
D-1
D-2
D-3
E-1
E-2
E-3
F-1
F-2
F-3

2.5 Effect of farnesol on planktonic inocula for biofilm formation by different *C. albicans* strains

Hornby medium is known to induce germ-tube formation in *C. albicans*. This experiment was designed to investigate biofilm formation by *C. albicans* prepared from overnight cultures grown on Hornby medium in the presence of 1 mM farnesol. Thus, overnight planktonic cultures were exposed to the effect of germ-tube enhancement by the growth medium and the effect of germ-tube inhibition by farnesol. Subsequently biofilm formation was determined in both the presence and absence of farnesol.

The effects of farnesol in this experiment were evaluated in *C. albicans* strains GDH 2346, CAI-4, H121 and H1. For biofilm formation, farnesol was added at adhesion only, or adhesion and time zero of biofilm formation. The results (Fig. 23) show that biofilm formation by *C. albicans* GDH 2346 was inhibited when farnesol was added at adhesion or adhesion and time zero by 29 and 33%, respectively ($P< 0.05$). In contrast, farnesol did not inhibit biofilm formation of the three other *C. albicans* strains (Fig. 23). Moreover, in further investigations farnesol showed similar activity when Hornby medium was replaced by YNB (Fig. 24); farnesol inhibited biofilm formation by 29 % and 33 % when added at adhesion, and at adhesion plus time zero of biofilm formation, respectively ($P<0.01$).

Figure 23

Effects of 1 mM farnesol on overnight cultures and biofilm formation by different *C. albicans* strains grown in Hornby medium

Biofilm inocula were prepared from overnight cultures grown in Hornby medium in the presence of 1 mM farnesol. Farnesol was added at adhesion, and at adhesion plus time zero of biofilm formation. Results are means ± SEM from two independent experiments carried out in triplicate. Mean (± SEM) control values (A_{492}) for different *C. albicans* strains ranged from 1.139 ± 0.058 to 2.065 ± 0.065.

Farnesol was added:

■ at adhesion

■ at adhesion plus time zero of biofilm formation

□ Control

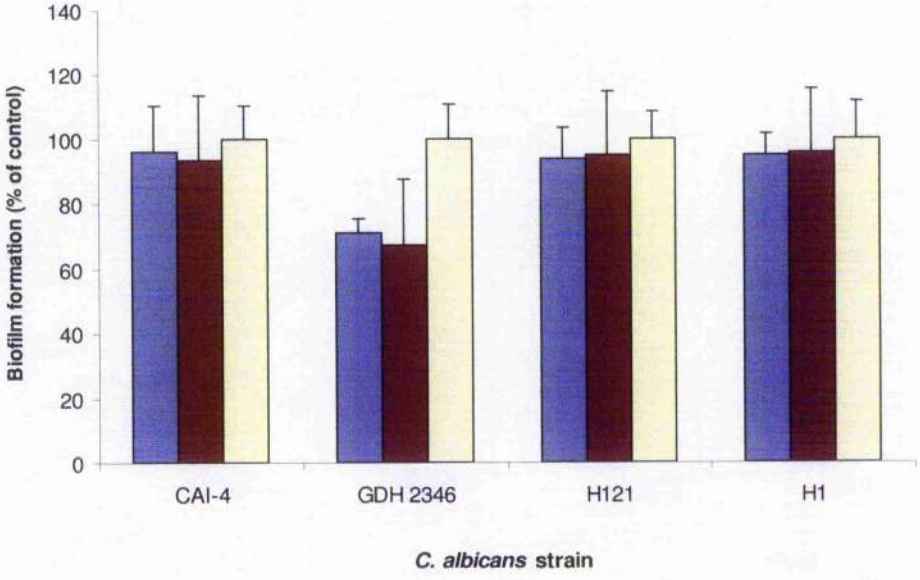
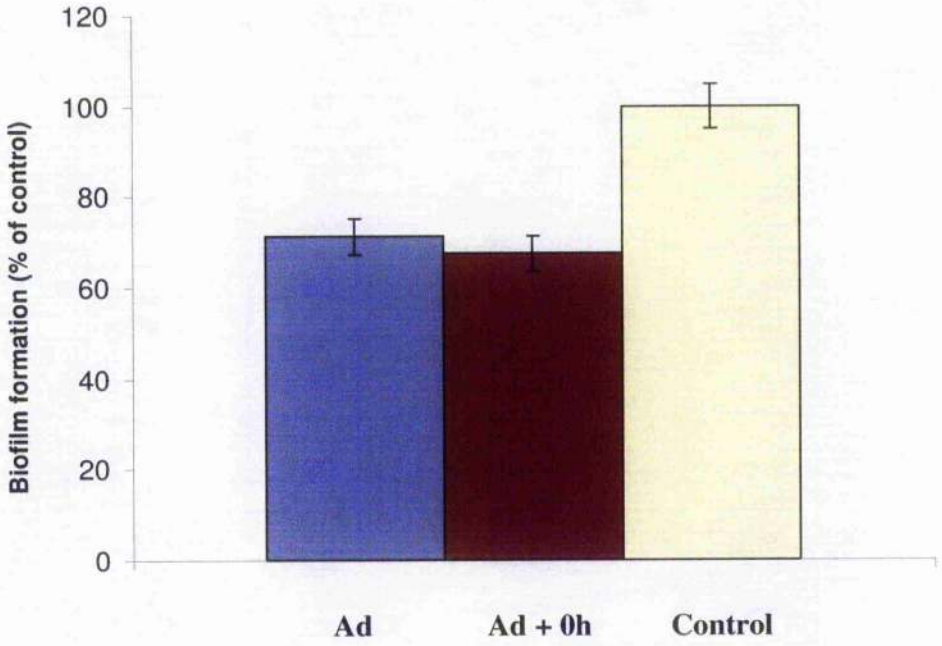

140
120
100
80
60
40
20
0
Biofilm formation (% of control)
CAI-4
GDH 2346
H121
H1
C. albicans strain

Figure 24

Effect of 1 mM farnesol on overnight cultures and biofilm formation of *C. albicans* GDH 2346 grown in YNB medium

Biofilm inocula were prepared from overnight cultures grown in YNB in the presence of 1 mM farnesol. Biofilms were grown in YNB, with 1 mM farnesol added at adhesion (Ad, ▩) or at adhesion plus time zero of biofilm formation (Ad+0h, ■). Results are means ± SEM from two independent experiments carried out in triplicate. Mean (± SEM) control value (A_{492}) for *C. albicans* GDH 2346 was 1.822 ± 0.093.


120
100
80
60
40
20
0
Biofilm formation (% of control)
Ad
Ad + 0h
Control

2.6 Biofilm formation in microtitre plates in the presence of farnesol

The results described above showed less inhibition by farnesol than those in an earlier study by Ramage *et al.* (2002) who demonstrated by a microtitre plate method that 300 μM farnesol inhibited biofilm formation by 85 % when added at time zero of biofilm formation (Ramage *et al.*, 2002a). In this experiment, the effect of farnesol on *C. albicans* biofilms was evaluated by the microtitre plate method i.e. biofilms were formed on the polystyrene surfaces of the plate wells. Biofilms were grown for 24 h in the presence or absence of 1 mM farnesol. Before biofilm metabolic activity was measured using the XTT assay, biofilms were washed in PBS three times, once or not at all (Fig. 25, A and B).

The results showed that farnesol inhibited biofilm formation by both *C. albicans* GDH 2346 and *C. albicans* CAI-4. For example, *C. albicans* GDH 2346 biofilm formation was inhibited by 13, 15 and 21% at no wash, one wash and three washes respectively ($P< 0.01$ to $P< 0.05$; Fig. 25 A). For biofilm formation by *C. albicans* CAI-4, the greatest inhibitory effect of farnesol was seen when biofilms were washed three times (38 %; $P<0.001$; Fig. 25 B). Overall the results show that there is a correlation between the XTT reduction observed and the number of washes. This suggests that biofilms grown in the presence of farnesol are more easily dislodged from the polystyrene surface.

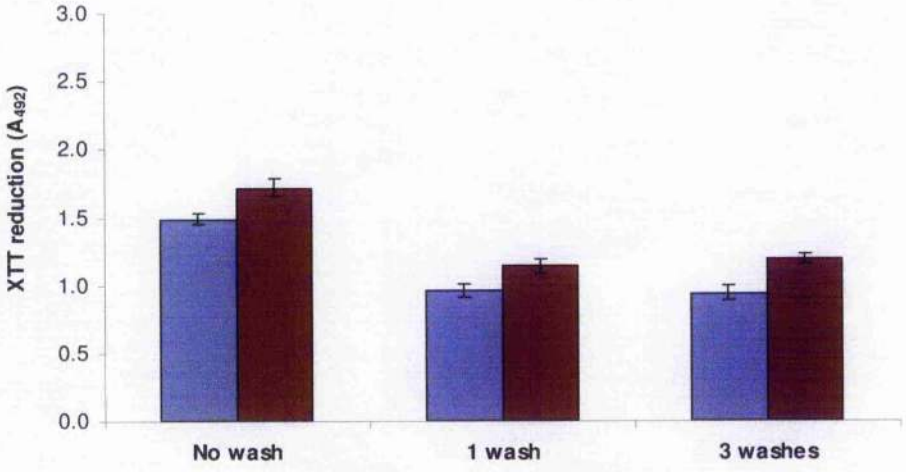
Figure 25

Effect of farnesol on biofilm formation in wells of microtitre plates

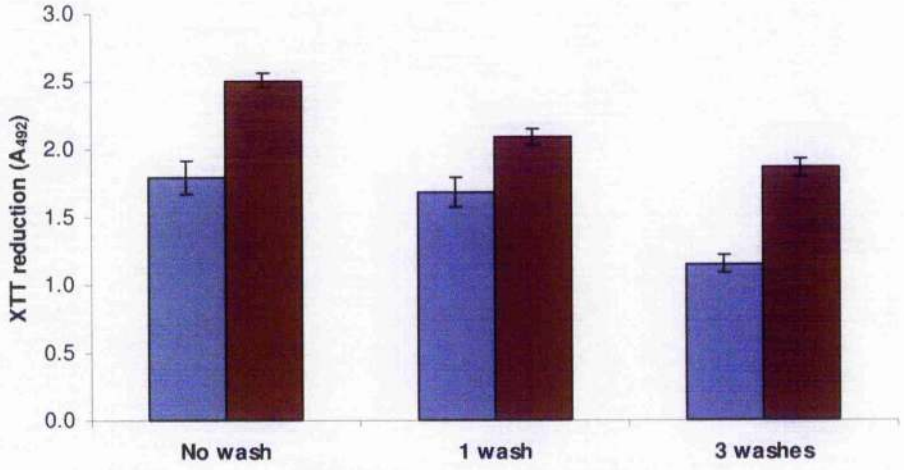
Biofilms were grown on the surfaces of wells of 96-well microtitre plates, in the presence or absence of 1 mM farnesol. Biofilm formation by *C. albicans* strain GDH 2346 (A) and strain CAI-4 (B) was measured using the XTT assay. Results are means ± SEM from two independent experiments with 16 replicates each time.

- Biofilm grown in the presence of 1 mM farnesol
- Control biofilm

A


3.0
2.5
2.0
1.5
1.0
0.5
0.0
XTT reduction (A492)
No wash
1 wash
3 washes

B


3.0
2.5
2.0
1.5
1.0
0.5
0.0
XTT reduction (A492)
No wash
1 wash
3 washes

2.7 Effect of different farnesol concentrations on biofilm formation by *C. albicans* strains obtained from the University of Nebraska

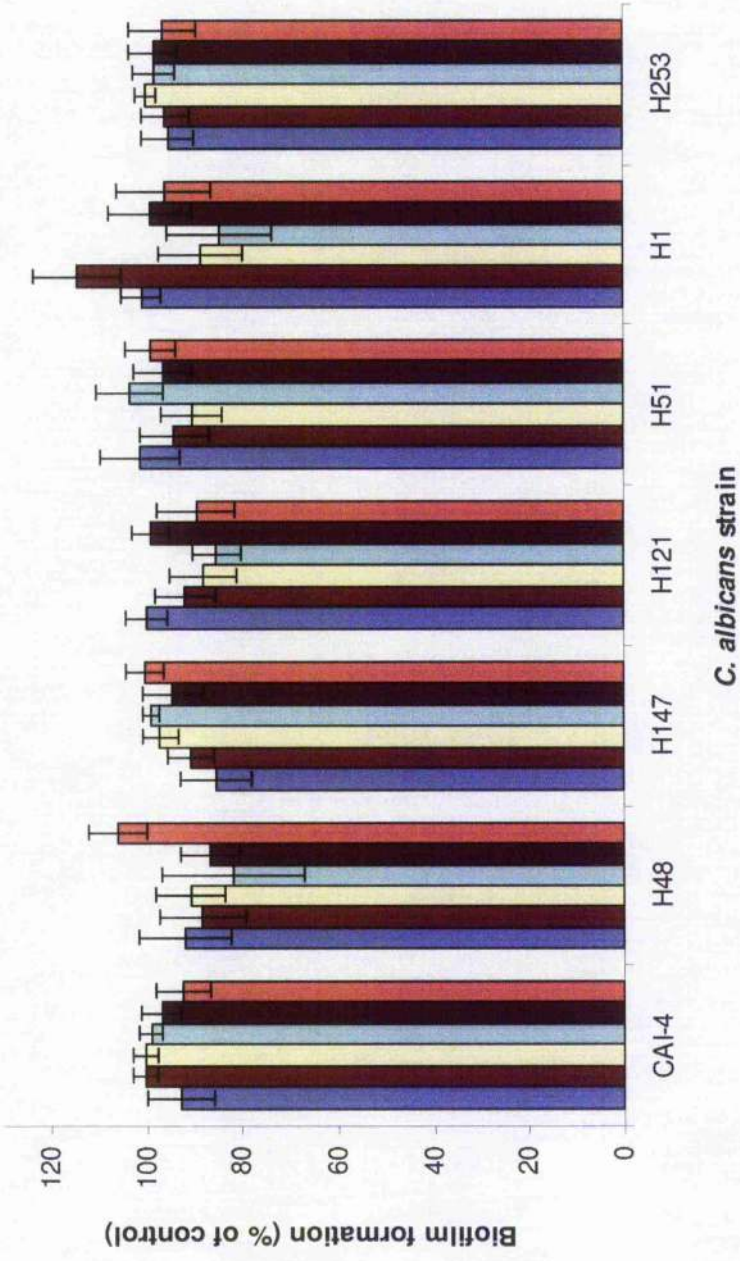
Previous results showed that 1 mM farnesol inhibited biofilm formation by *C. albicans* GDH 2346 when added for the adhesion period and at time zero of biofilm formation. In this experiment, the effects of farnesol were further investigated in another seven *C. albicans* strains obtained from the University of Nebraska. These strains are mutants with different colony morphologies and different responses to farnesol. Three farnesol concentrations (50 μM, 100 μM or 1 mM) were added for the adhesion period only, or for adhesion and at time zero of biofilm formation. The effects of farnesol were determined by subsequent measurement of biofilm metabolic activity. The results (Fig. 26) appeared to show some inhibition by farnesol with some *C. albicans* strains, but none of these results were statistically significant ($P>0.05$).

Figure 26

Effect of three different farnesol concentrations on biofilm formation by *C. albicans* mutants obtained from the University of Nebraska

Biofilm formation by the *C. albicans* strains (as measured by XTT reduction) is expressed as a percentage of that of control biofilms incubated in the absence of farnesol. Farnesol (50 μM, 100 μM or 1 mM) was added at adhesion (Ad) or adhesion and time zero (Ad + 0h) of biofilm formation. Results are means ± SEM from two independent experiments with at least five replicates each time. Mean (± SEM) control values (A_{492}) for all the strains ranged from 2.479 ± 0.405 to 3.113 ± 0.260.

- 50 μM (Ad)
- 50 μM (Ad + 0h)
- 100 μM (Ad)
- 100 μM (Ad + 0h)
- 1 mM (Ad)
- 1 mM (Ad + 0h)


Biofilm formation (% of control)
120
100
80
60
40
20
0
CAI-4
H48
H147
H121
H51
H1
H253
C. albicans strain

2.8 Scanning electron microscopy of biofilm formation by *C. albicans* Nebraska strains in the presence of 1 mM farnesol

Scanning electron microscopy was used in this investigation to determine whether 1 mM farnesol could affect the biofilm structure of these *C. albicans* strains, since the previous results showed that farnesol did not affect biofilm metabolic activity. Initially, scanning electron microscopy showed that control biofilms of most of the strains consisted almost exclusively of hyphae (Fig.27), apart from biofilms of *C. albicans* CAI-4 and H121 which consisted of a mixture of yeasts and hyphae (Fig. 27 A-1 and D-1). In the presence of farnesol, *C. albicans* CAI4, H121 and H253 formed yeast-only biofilms (Fig. 27 A-2, D-2 and G-2), while biofilms of strains H1 and H147 consisted mostly of yeasts with only occasional hyphae (Fig. 27, C-2 and F-2). On the other hand, biofilms of *C. albicans* H48 and H51 retained their mainly hyphal structure (Fig. 27 B-2 and E-2).

Figure 27

Scanning electron micrographs of biofilm formation by *C. albicans* mutants obtained from the University of Nebraska

Biofilms were grown on PVC catheter disks for 48h in the absence of farnesol (1), or in 1 mM farnesol added for the adhesion period and at time zero of biofilm formation (2). Bars, 8 μm (1) and 16 μm (2).

A. *C. albicans* CAI-4
B. *C. albicans* H48
C. *C. albicans* H147
D. *C. albicans* H121
E. *C. albicans* H51
F. *C. albicans* H1
G. *C. albicans* H253

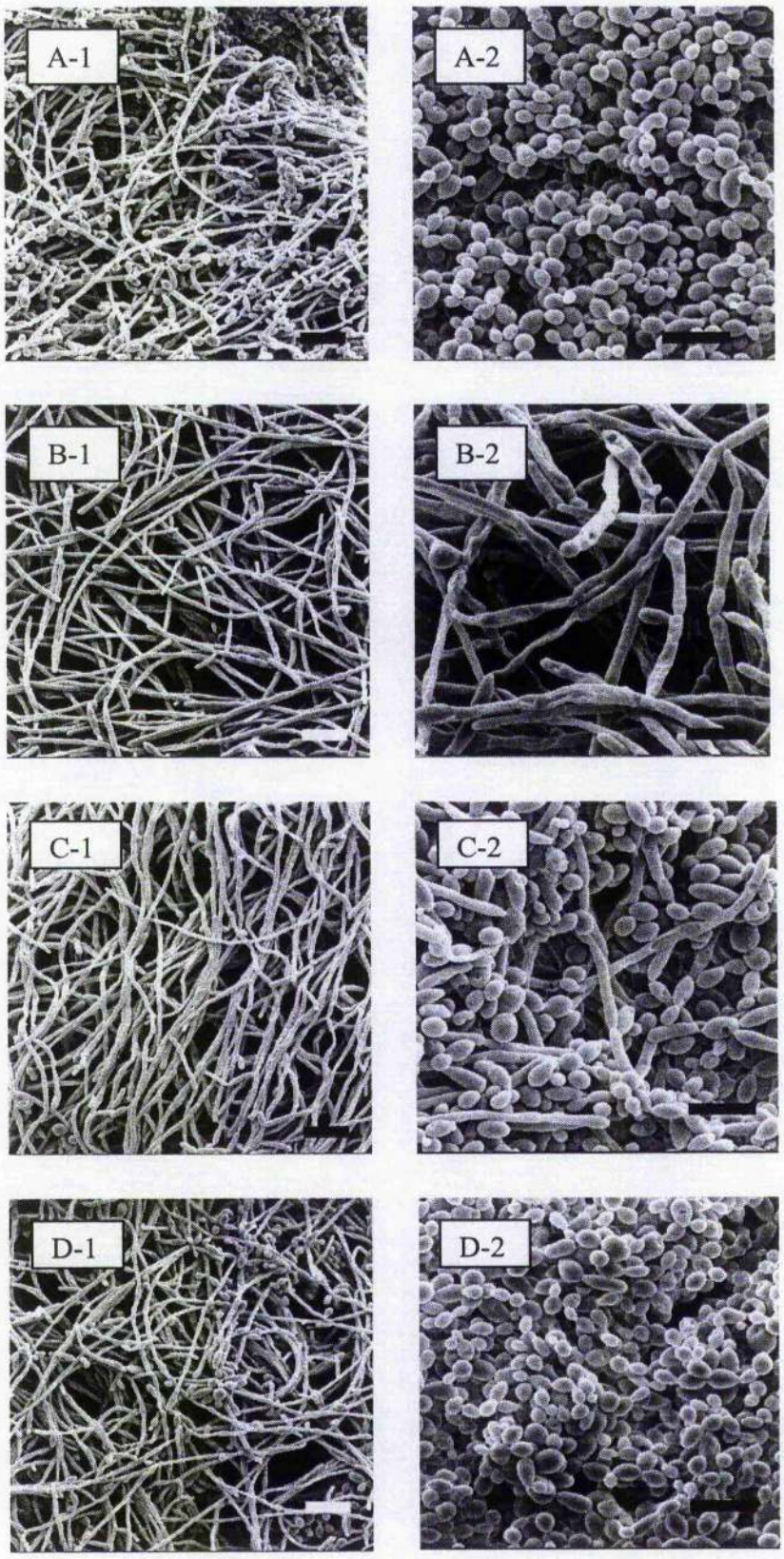
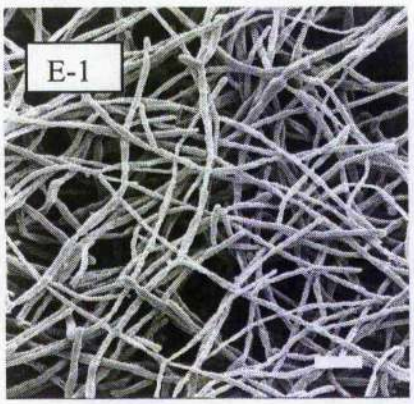
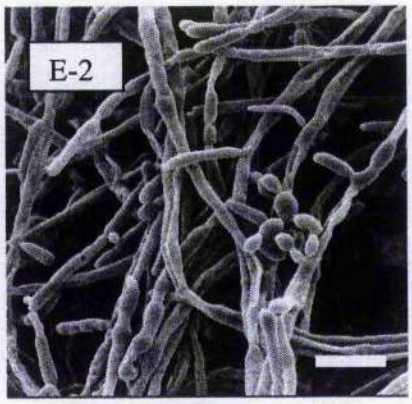
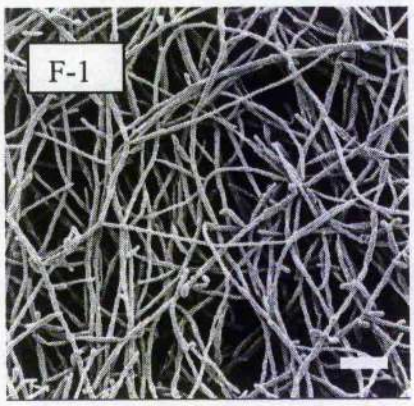

A-1
A-2
B-1
B-2
C-1
C-2
D-1
D-2

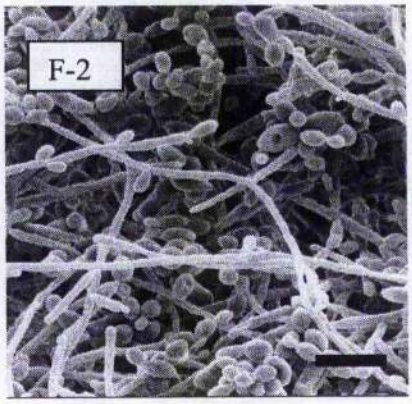
Figure 27

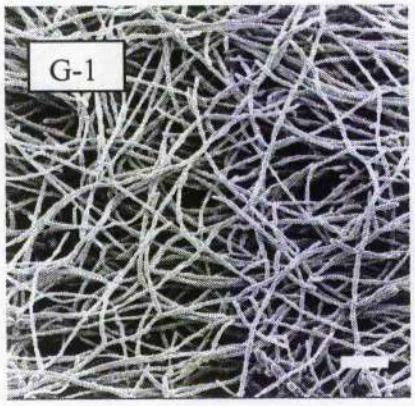
(Continued)

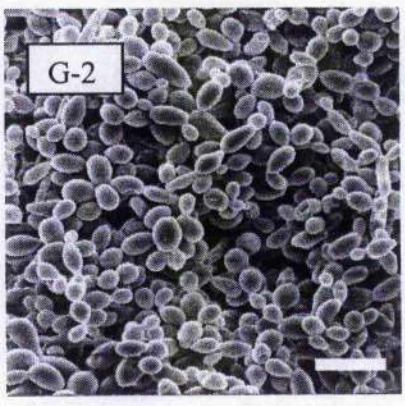

E-1


E-2


F-1


F-2


G-1


G-2

2.9 Effect of biofilm culture supernatants on the morphology of planktonic cells

A previous report by Hornby *et al.*(2001) showed that quorum-sensing molecules could be isolated from culture supernatants of planktonic cells of *C. albicans* (Hornby *et al.*, 2001). Another report confirmed that this quorum-sensing activity was also present in supernatants obtained from *C. albicans* biofilms (Ramage *et al.*, 2002a). It was therefore of interest to investigate quorum-sensing activity in biofilm supernatants of some of the *C. albicans* strains used in this study. Initially, planktonic cells were grown in RPMI 1640 medium in the presence of 100 μM farnesol. Biofilm supernatants were prepared from biofilms of the same strain grown in RPMI 1640 medium in 75-cm^2 tissue culture flasks as described by Ramage *et al* (2002a). Control planktonic cells were grown in RPMI 1640 without farnesol. The results (Fig. 28) showed that hypha formation was inhibited by 51 to 88% for most *C. albicans* strains when planktonic cells were treated with biofilm supernatant; this inhibition was statistically significant ($P<0.05$ to $P<0.01$). Farnesol (100 μM) added to cultures of planktonic cells inhibited hypha formation more than did homologous biofilm supernatant; 88 to 97% of hypha formation was inhibited for all *C. albicans* strains ($P<0.01$ to $P<0.001$) (Fig. 28). In a further experiment, planktonic cells of *C. albicans* CAI-4 were grown with biofilm supernatant obtained from different *C. albicans* strains. The results showed that most biofilm supernatants inhibited hypha formation by *C. albicans* CAI-4 by more than 70% ($P<0.001$; Fig. 29).

Figure 28

Effect of biofilm supernatant on hypha formation by planktonic cells using different *C. albicans* strains

Planktonic cells were grown for 24h at 37 °C in: (i) 20 ml RPMI 1640 as a control; (ii) 20 ml filter-sterilized biofilm supernatant diluted 1:1 with a two-fold concentrate of RPMI 1640 (■); and (iii) 20 ml RPMI 1640 with farnesol at a final concentration of 100 μM (■). Hypha formation is expressed as percentage of that of control planktonic cells incubated in RPMI 1640 in the absence of farnesol. Results are means ± SEM of two independent experiments. Mean (± SEM) control values ranged from 50 ± 5 to 85 ± 4 hyphae / 100 cells counted. Planktonic cells were incubated for 24h at 37 °C, and then the numbers of yeast cells and hyphae were determined by light microscopy.

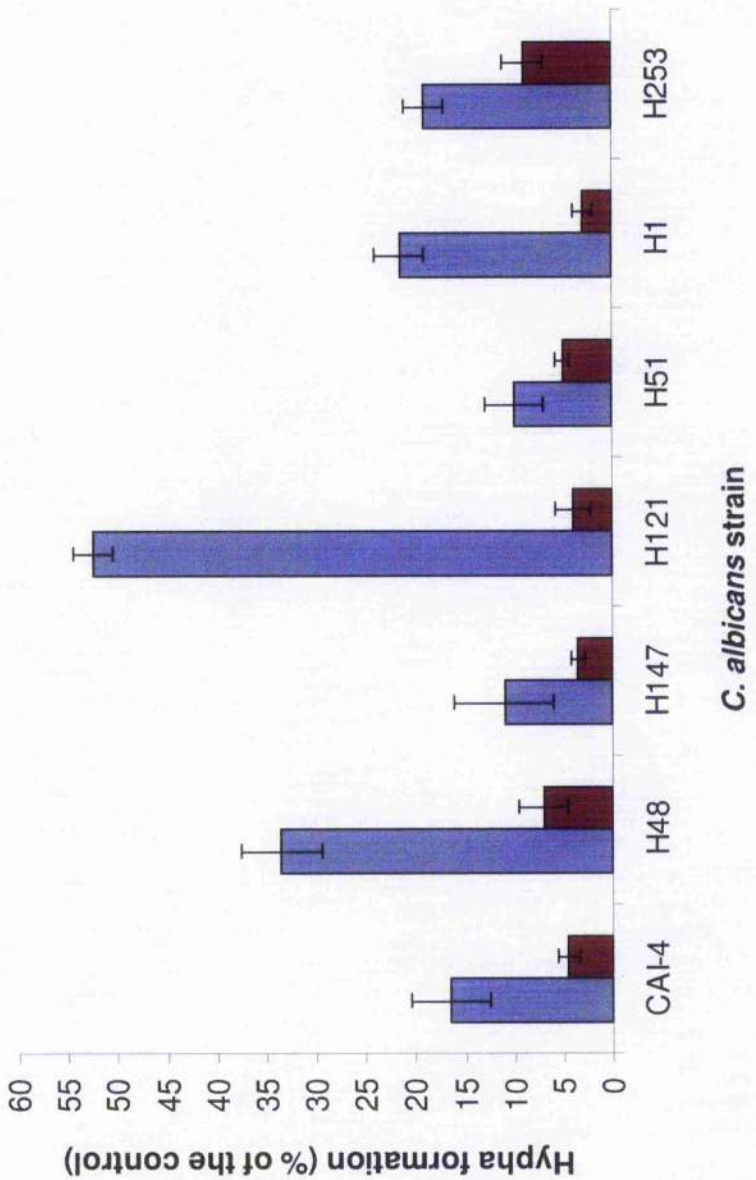
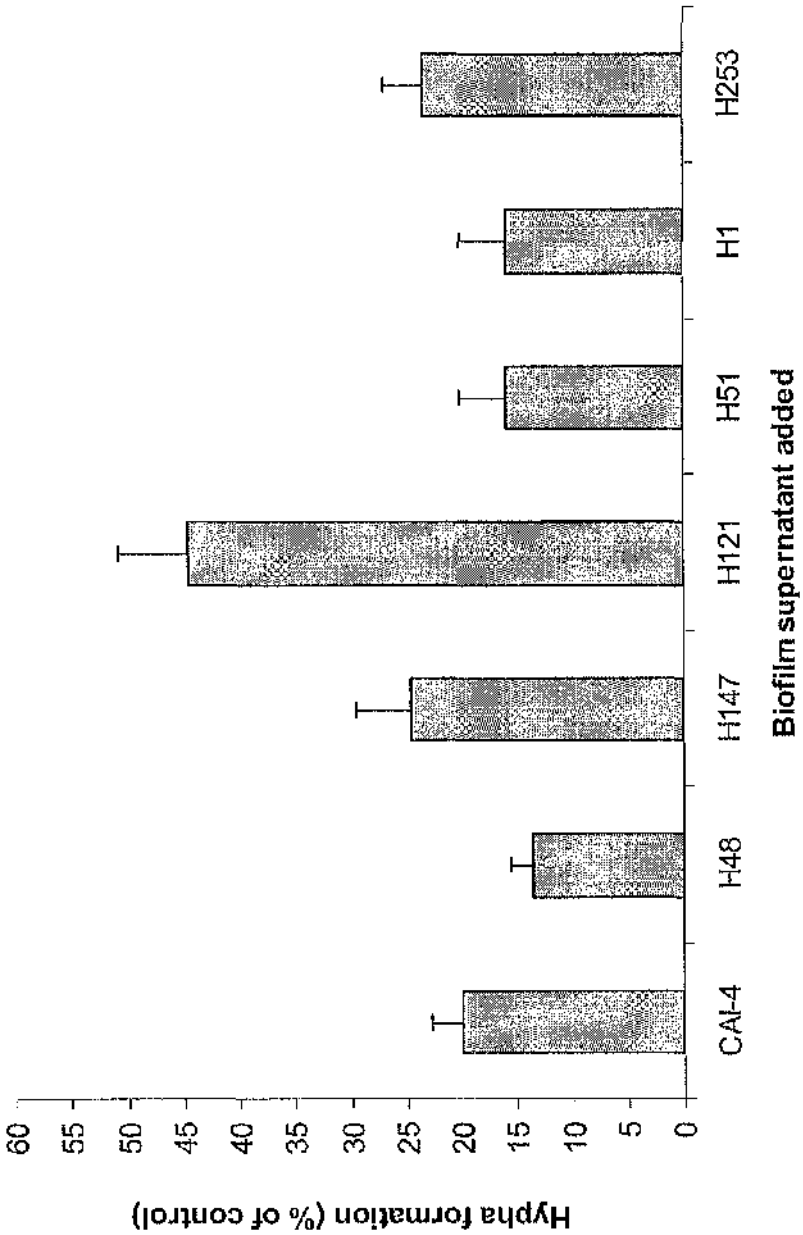

Hypha formation (% of the control)
60
55
50
45
40
35
30
25
20
15
10
5
0
CAI-4
H48
H147
H121
H51
H1
H253
C. albicans strain

Figure 29

Effect of different biofilm supernatants on planktonic cells of *C. albicans* CAI-4

Hypha formation is expressed as a percentage of that of control planktonic cells of *C. albicans* CAI-4 incubated in RPMI 1640 in the absence of biofilm supernatant. Results are means ± SEM of two independent experiments. The mean (± SEM) control value was 84 ± 5 hyphae / 100 cells counted.


Hypha formation (% of control)
60
55
50
45
40
35
30
25
20
15
10
5
0
CAI-4
H48
H147
H121
H51
H1
H253
Biofilm supernatant added

2.10 Effect of planktonic and biofilm culture supernatants on germ-tube formation by *C. albicans*

To investigate the concentration of farnesol (and possibly other, similar quorum-sensing molecules) produced during planktonic cell growth and biofilm formation, culture supernatants of *C. albicans* SC5314 were collected at 24, 48 and 72h and tested for their ability to inhibit germ-tube formation by the same *C. albicans* strain. This was accomplished by measuring cell dry weights at the times that supernatant samples were collected, and by determining the percentage inhibition of germ-tube formation produced by the supernatants. The results showed that supernatants from planktonic cells after growth for 24, 48 and 72h inhibited germ-tube formation by 7, 17 and 20 %, respectively (Fig. 30 A). However, supernatants from biofilms harvested at the same time points inhibited germ-tube formation by 10, 28 and 42% (Fig. 30 B).

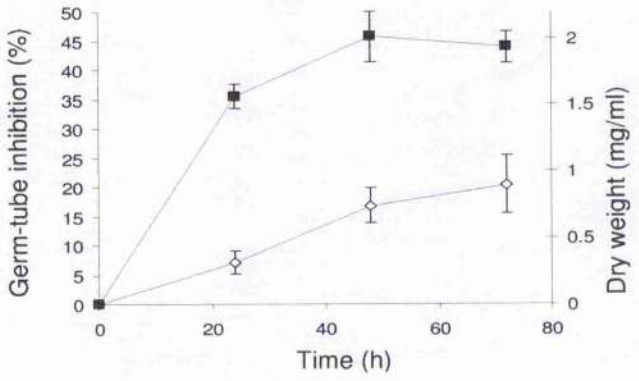
When the percentage inhibition was calculated as a function of cell dry weight, it became apparent that biofilm cells produce significantly more farnesol (and possibly other, similar quorum-sensing molecules) than planktonic cells. For example, supernatant equivalent to 1mg dry wt of biofilms formed at 24, 48 and 72h inhibited germ-tube formation by 11, 28 and 47 %, respectively. In contrast, supernatant from 1mg dry wt of planktonic cells inhibited germ-tube formation by only 5, 8 and 11% (Fig. 30 C). This suggests that the production of farnesol (and possibly other, similar quorum-sensing molecules) increases more during the later stages of biofilm formation than it does during planktonic cell growth.

Figure 30

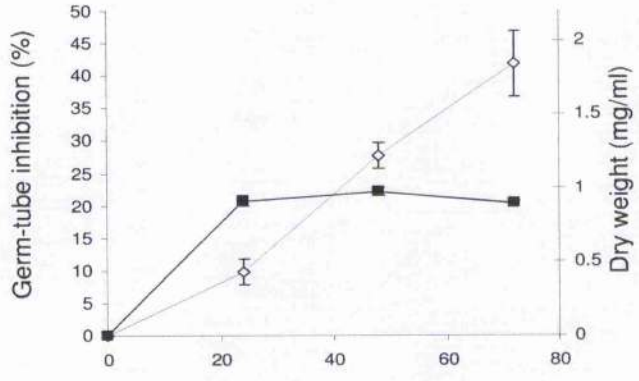
Effects of planktonic and biofilm culture supernatants on germ-tube formation by *C. albicans* SC5314

The inhibitory effect of culture supernatants on germ-tube formation (◇) is shown together with cell dry weight over 72h (■) for planktonic cells (A) and biofilms (B). This percentage inhibition of germ-tube formation is also shown as a function of cell dry weight (C) for planktonic cultures (■) and biofilms (■). Germ-tube formation was determined as a percentage of that for control cells incubated in the absence of culture supernatants. Results are means ± SEM of two independent experiments carried out in triplicate. Mean (± SEM) values for the controls ranged from 158 ± 7 to 180 ± 12 cells/200 cells counted.

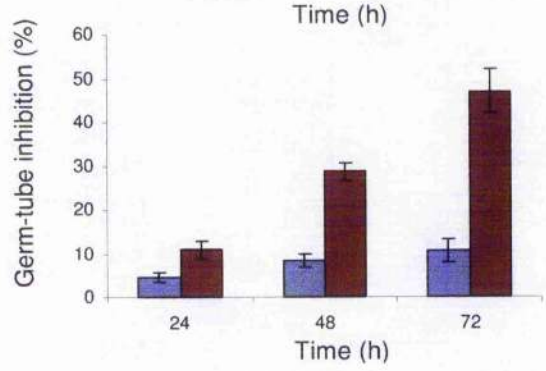
A


Germ-tube inhibition (%)
Dry weight (mg/ml)
Time (h)
0
5
10
15
20
25
30
35
40
45
50
0
0.5
1
1.5
2
0
20
40
60
80

B


Germ-tube inhibition (%)
Dry weight (mg/ml)
Time (h)
0
5
10
15
20
25
30
35
40
45
50
0
0.5
1
1.5
2
0
20
40
60
80

C


Germ-tube inhibition (%)
Time (h)
0
10
20
30
40
50
60
24
48
72

2.11 Biofilm formation by morphological mutants in the presence of 1 mM farnesol or propranolol

In order to further investigate the activity of farnesol as a quorum-sensing molecule, other *C. albicans* strains were evaluated, including *C. albicans* SC5314 and its morphological mutants described earlier. Moreover, in these experiments the effect of propranolol was studied to determine whether biofilm formation by the morphological mutants which are blocked in signalling pathways (*efg1/efg1* or *cph1/cph1* or *efg1/efg1 cph1/cph1*) could be inhibited by 1 mM propranolol or 1 mM farnesol when these compounds were added at the beginning of adhesion period and again at time zero of biofilm formation. As can be seen from Table 4, neither farnesol nor propranolol affected biofilm formation by these strains as compared with control biofilms grown in the absence of farnesol or propranolol.

2.12 Scanning electron microscopy of biofilm formation by *C. albicans* SC5314 and its morphological mutants in the presence of farnesol or propranolol

The results in section 2.11 showed that biofilm metabolic activity of *C. albicans* strains blocked in signalling pathways was unaffected in the presence of 1 mM farnesol or 1 mM propranolol. Here, biofilm formation by these strains was examined by Scanning electron microscopy. This was important since other investigators reported that such mutants fail to produce any biofilms (Lewis *et al.*, 2002; Ramage *et al.*, 2002b). The results (Fig. 31) show that control biofilms of strains SC5314 and JKC19 consisted of a mixture of yeasts and hyphae (Fig. 31, A-1 and B-1), whereas in the presence of farnesol or propranolol biofilms of these two strains contained a high proportion of yeast cells (Fig. 31, A-2,3 and B-

2,3). On the other hand, strains HLC52 and HLC54 formed yeast-only biofilms in the presence or absence of farnesol or propranolol (Fig. 31, C-1,2,3 and D-1,2,3). However, the biofilms produced by these strains were extensive and contained densely packed yeast cells, in the contrast to the earlier reports.

2.13 Biofilm formation by *C. albicans* CAF2-1 and its quorum sensing mutant strains lacking *CHK1*

It has been shown that a *C. albicans* Chk21 mutant, lacking the Chk1p histidine kinase, is refractory to the inhibitory effect of farnesol. For example, in the presence of farnesol, biofilm formation by the wild type, *C. albicans* CAF2-1, was reduced, whereas biofilm formation by the Chk21 strain was not affected (Kruppa *et al.*, 2004). Since these results were obtained using a microtitre plate assay, it was important to monitor biofilm formation by these strains at different time periods using the more reliable catheter disk assay to determine any differences between the mutant and wild-type strains. No significant differences were observed between biofilms formed by *C. albicans* CAF2-1 and the two mutant strains up to 24h of incubation (Fig. 32). However, from 48 to 72h *C. albicans* Chk21 (*chk1/chk1*) showed significantly increased biofilm formation compared with *C. albicans* CAF2-1 and Chk23 (*Chk1/CHK1*) ($P<0.001$ to $P<0.01$, respectively; Fig. 32). For example, XTT reduction (A_{492}) at 48h for strain Chk21 was 3.039 ± 0.048, whereas for strains CAF2-1 and Chk23 A_{492} values were nearly similar at 2.670 ± 0.046 and 2.678 ± 0.032, respectively (Fig. 32).

Table 4. Biofilm formation of *C. albicans* SC5314 and its morphological mutants with farnesol and propranolol

C. albicans strain	XTT reduction (%) [a] with 1 mM	
	Farnesol	Propranolol
SC5314	93 ± 4.5	102 ± 1.0
JKC 19 (*cph1*)	93 ± 2.6	101 ± 0.3
HLC 52 (*efg1*)	102 ± 1.2	98 ± 0.9
HLC 54 (*cph1/efg1*)	97 ± 4.1	97 ± 0.7

[a] Biofilm formation is expressed as a percentage of that control biofilms incubated in the absence of farnesol or propranolol. The results are means ± SEM from one experiment with three replicates. Farnesol and propranolol were added at adhesion and time zero of biofilm formation, Mean (± SEM) control values (A_{492}) for all the strains ranged from 2.932 ± 0.033 to 3.298 ± 0.024.

Figure 31

Scanning electron micrographs of biofilm formation by *C. albicans* SC5314 and its morphological mutants in the presence of farnesol and propranolol

Biofilms were grown on PVC catheter disks for 48h. The figure shows control biofilms (1) and biofilms grown in the presence of 1 mM farnesol (2) or 1 mM propranolol (3) for the strains listed below. Bar, 10 μm.

A. *C. albicans* SC5314

B. *C. albicans* JKC 19 (*cph1/cph1*)

C. *C. albicans* HLC 52 (*eph1/eph1*)

D. *C. albicans* HLC 54 (*cph1/cph1 efg1/efg1*)

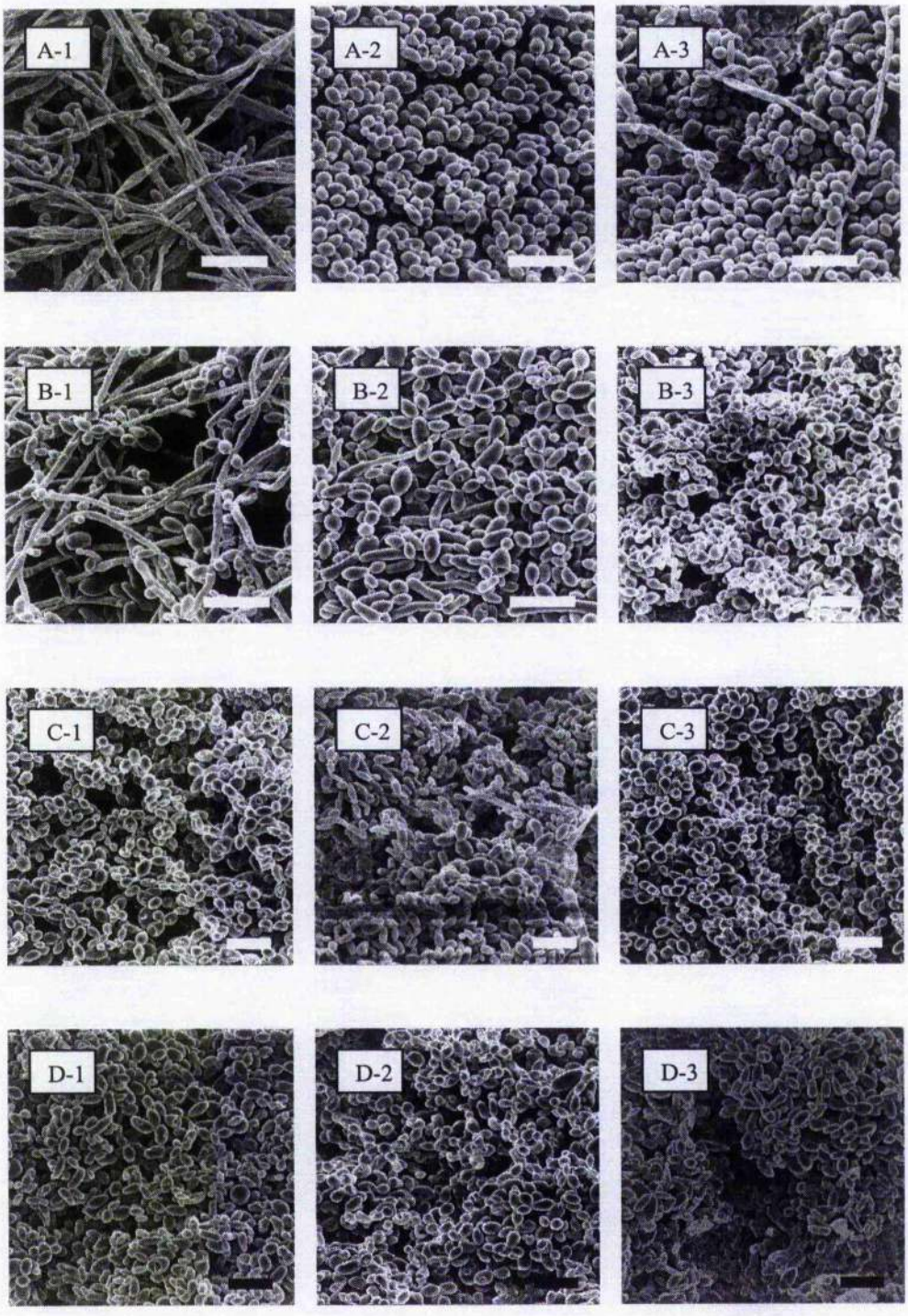

A-1
A-2
A-3
B-1
B-2
B-3
C-1
C-2
C-3
D-1
D-2
D-3

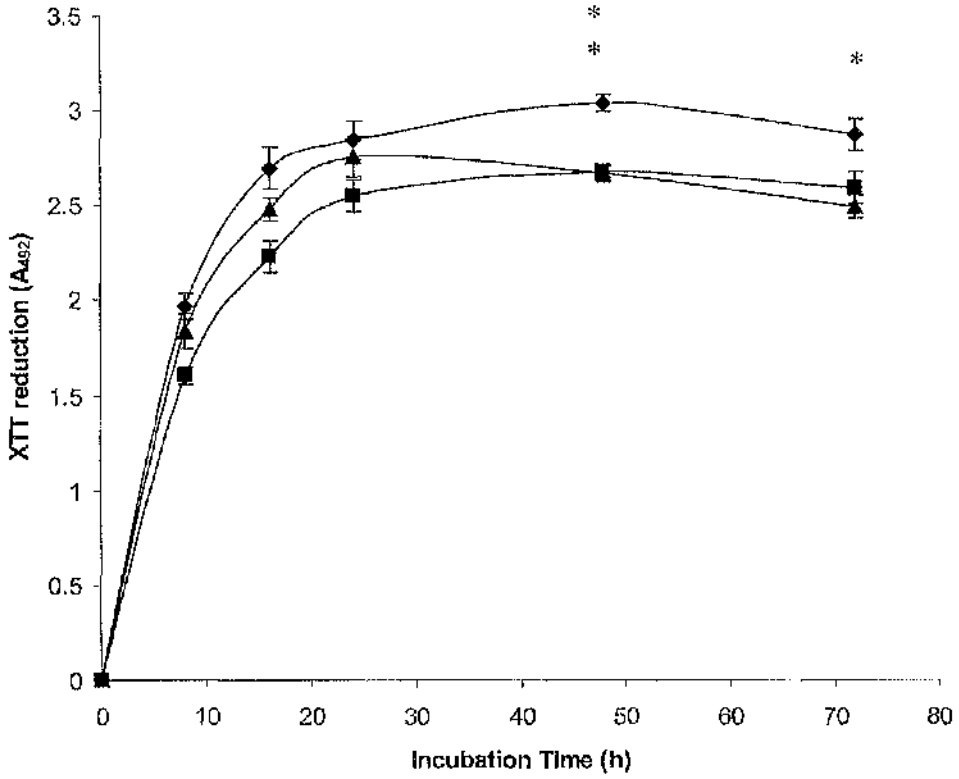
Figure 32

Biofilm formation by *C. albicans* CAF2-1 and its quorum sensing mutant strains lacking Chk1p

Biofilms of *C. albicans* CAF2-1 (▲), Chk21 (◆) and Chk23 (■) were grown on PVC catheter disks for 72h at 37°C. At 0, 8, 16, 24, 48 and 72h, biofilm formation was measured as cell metabolic activity (XTT reduction at A_{492}). The results are means ± SEM from two independent experiments done in quadruplicate.

* $P < 0.01$ for biofilms of strain Chk21 compared with those of strain CAF2-1 or Chk23

* * $P < 0.001$ for biofilms of strain Chk21 compared with those of strain CAF2-1 or Chk23


3.5
3
2.5
2
1.5
1
0.5
0
XTT reduction (A_{492})
0
10
20
30
40
50
60
70
80
Incubation Time (h)
*
*
*

3. Role of tyrosol as a quorum-sensing molecule in *C. albicans* biofilms

Tyrosol was recently identified in planktonic cultures of *C. albicans* as another type of quorum-sensing molecule which promotes hyphal formation and reduces the lag phase before exponential growth is initiated (Chen *et al.*, 2004). However, the role of tyrosol in regulating *C. albicans* biofilm formation is unknown. In this section, the effects of tyrosol on biofilm development, and tyrosol production by planktonic and biofilm cells are described.

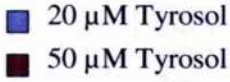
3.1 Biofilm formation in the presence of tyrosol

Here, the effect of tyrosol on biofilm formation by *C. albicans* GDH 2346 was investigated. Tyrosol at 20 μM or 50 μM was added at different stages (0, 3 , 6 , 24 and 48 h) of biofilm formation, and biofilm metabolic activity was determined after 48h. The results (Fig. 33) show biofilm formation of *C. albicans* GDH 2346 was not affected by either tyrosol concentration added at an early or late stage. For example, 20 or 50 μM tyrosol added at time zero or at 24h produced biofilms with a metabolic activity similar to that of untreated biofilms (Fig 33). In a separate experiment the effect of higher tyrosol concentrations (100 μM, 500 μM and 1000 μM) added at an early stage was investigated with biofilms grown on YNB or RPMI 1640 medium, The results showed that tyrosol did not significantly affect biofilms grown in either medium at any of these concentration (Fig. 34A and B).

Figure 33

Effect of tyrosol on biofilm formation by *C. albicans* GDH 2346

Biofilm formation of *C. albicans* (as measured by XTT reduction) is expressed as a percentage of that of control biofilms incubated for 48h in the absence of tyrosol. Tyrosol at 50 μM or 100 μM was added at 0, 3, 6, 24 and 48h of the incubation. Results are means ± SEM from two independent experiments done in triplicate. The mean (± SEM) control value (A_{492}) was 2.271± 0.061.



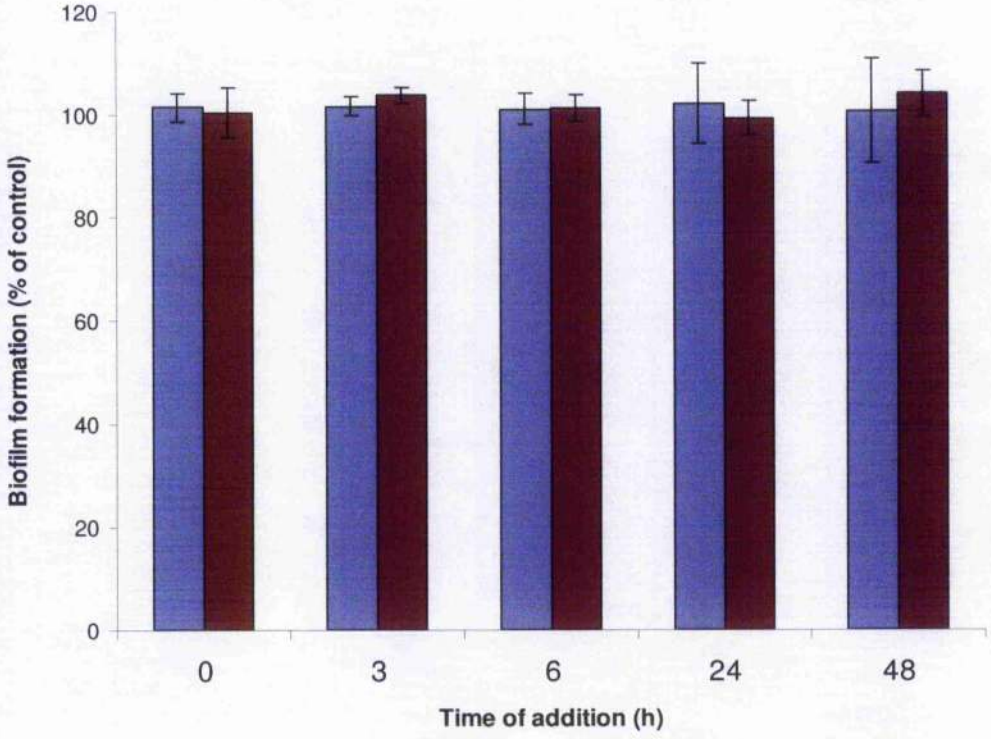
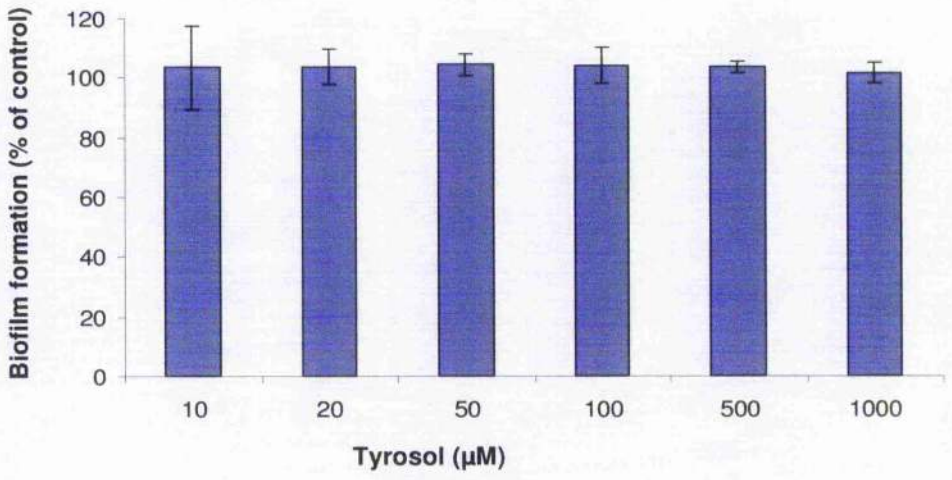

120
100
80
60
40
20
0
Biofilm formation (% of control)
0
3
6
24
48
Time of addition (h)

Figure 34

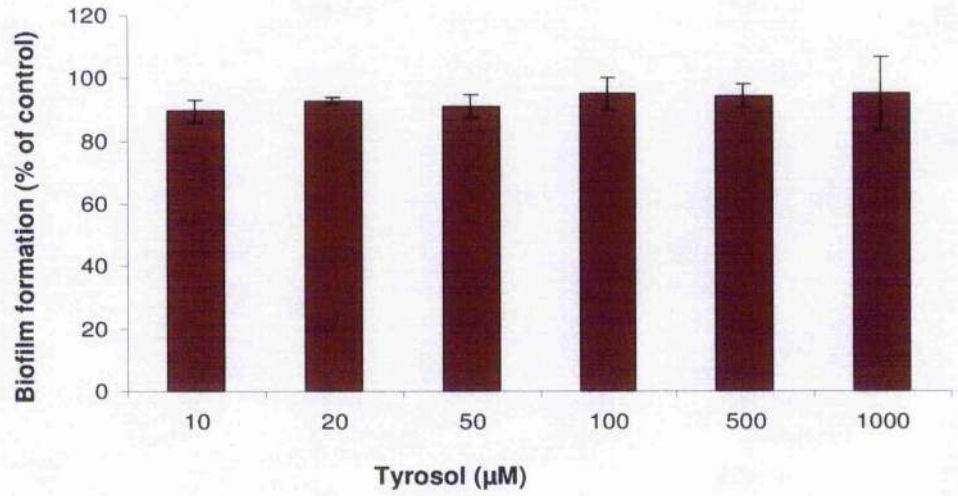
Effect of different tyrosol concentrations on biofilm formation by *C. albicans* GDH 2346 grown in YNB or RPMI 1640 medium

Biofilms were grown in YNB (A) or RPMI 1640 medium (B). Tyrosol was added at time zero of biofilm formation. Biofilm formation after 48h is expressed as a percentage of that of control biofilms incubated in the absence of tyrosol. Results are means ± SEM from two independent experiments done in triplicate. Mean (± SEM) control values (A_{492}) for biofilms grown in YNB and RPMI 1640 were 0.871 ± 0.032 and 1.967 ± 0160 respectively. XTT reduction in this experiment was measured after 3h rather than 5h, so that the A_{492} values for biofilms grown in RPMI 1640 medium were less than 3.500.

A


Biofilm formation (% of control)
120
100
80
60
40
20
0
10
20
50
100
500
1000
Tyrosol (µM)

B


Biofilm formation (% of control)
120
100
80
60
40
20
0
10
20
50
100
500
1000
Tyrosol (µM)

3.2 Scanning electron microscopy of the effect of tyrosol on biofilm formation by *C. albicans* GDH 2346

To determine the effect of tyrosol on *C. albicans* biofilm structure, tyrosol at three concentrations (50, 500 and 1000 μM) was added at time zero of biofilm formation. Scanning electron microscopy demonstrated that that tyrosol-treated biofilms at all tyrosol concentrations (Fig 35 B, C and D) had a structure similar to that of control biofilms i.e. a mixture of yeasts and hyphae (Fig. 35 A). Thus, tyrosol addition did not affect biofilm structure when examined after 48h of incubation.

3.3 Scanning electron microscopy of the effect of tyrosol on biofilm formation by *C. albicans* SC5314 and its morphological mutants

The effect of tyrosol on biofilm structure was further investigated using *C. albicans* SC5314 and its morphological mutants, to determine whether tyrosol could stimulate hyphal formation in *C. albicans* strains defective in the signalling pathways containing efg1 or cph1. Strains JKC 19 (*cph1*), HLC 52 (*efg1*) and HLC 54 (*cph1/efg1*) were used together with the wild-type strain *C. albicans* SC5314 in these experiments. Biofilm formation was carried out in the presence of 20 and 50 μM tyrosol. Scanning electron micrographs (Fig. 36 C-1,2,3 and D-1,2,3) showed that control biofilms and tyrosol-treated biofilms of strains HLC 52 (*efg1*) and HLC 54(*cph1/efg1*) contained yeast cells exclusively, indicating that tyrosol was not able to stimulate hyphal formation in *C. albicans* defective in the efg1 pathway. Biofilm structure in *C. albicans* SC5314 and JKC 19 was similar, and consisted of a mixture of yeasts and hyphae in the presence or absence of tyrosol (Fig. 36, A-1, 2, 3 and B-1, 2, 3).

Figure 35

Scanning electron micrographs showing the effect of tyrosol on *C. albicans* GDH 2346 biofilms grown in YNB

Tyrosol was added at time zero of biofilm formation. *C. albicans* GDH 2346 biofilms were grown on PVC disks for 48h. (A) Control biofilm; (B) with 50 μM tyrosol; (C) with 500 μM; (D) with 1 mM tyrosol. Bar, 10 μm.

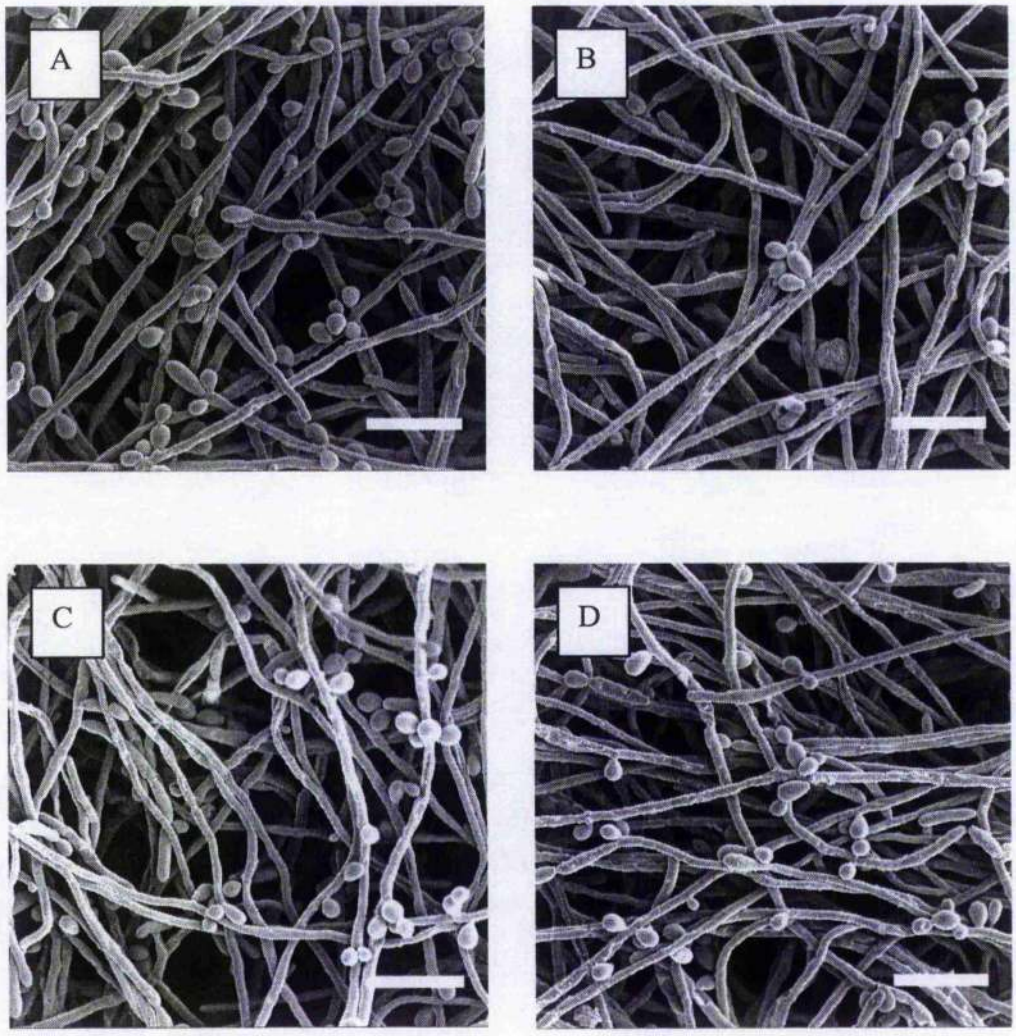

A
B
C
D

Figure 36

Effect of tyrosol on biofilm structure of *C. albicans* SC5314 and its morphological mutants

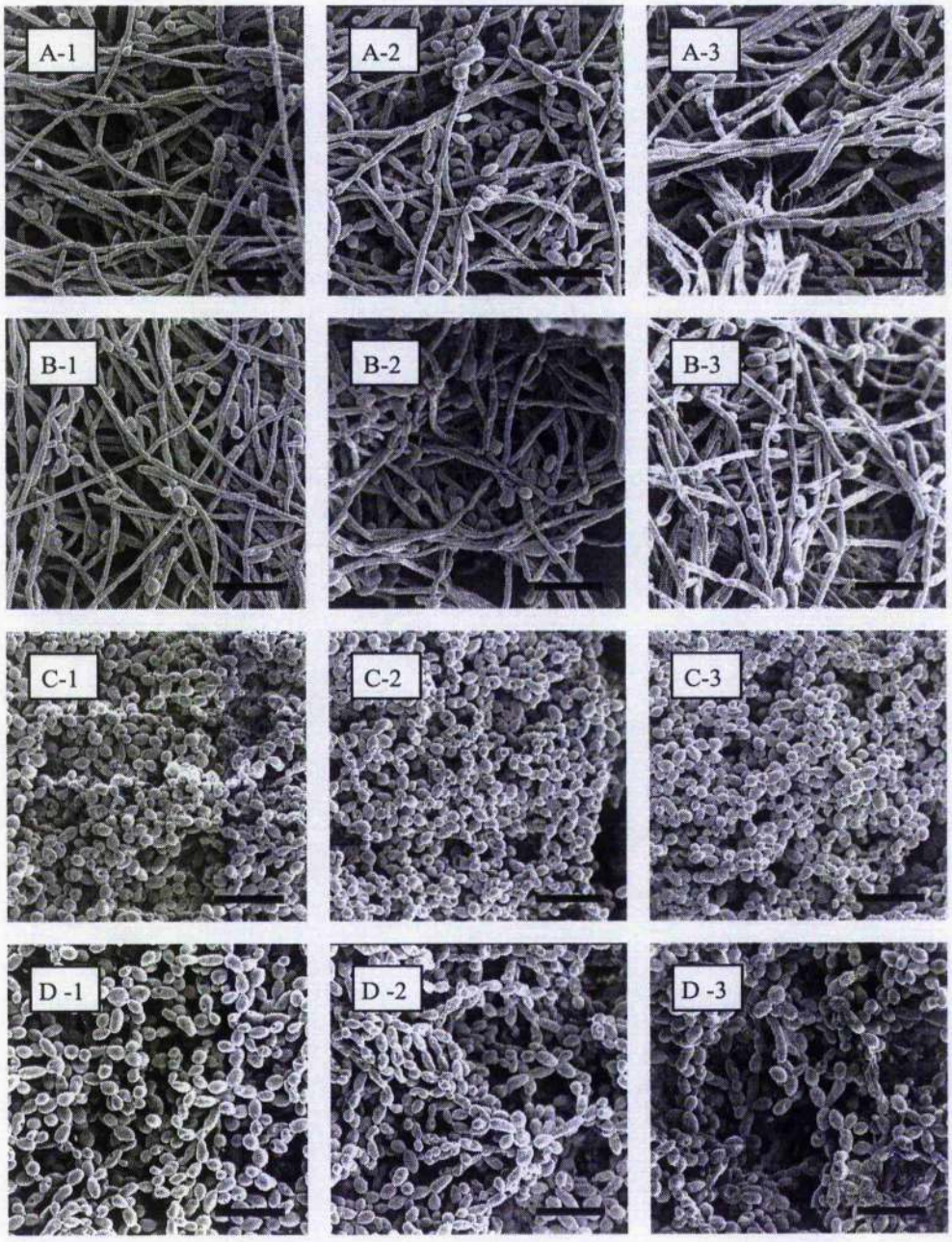
Biofilms were formed on catheter disks incubated for 48h in the presence of 20 μM (2) or 50 μM tyrosol (3). Control biofilms were formed in the absence of tyrosol (1). Bar, 8 μm.

A. *C. albicans* SC5314

B. *C. albicans* JKC 19 (*cph1/cph1*)

C. *C. albicans* HLC 52 (*efg1/efg1*)

D. *C. albicans* HLC 54 (*cph1/cph1 efg1/efg1*)


A-1
A-2
A-3
B-1
B-2
B-3
C-1
C-2
C-3
D -1
D -2
D -3

3.4 Tyrosol production by planktonic cells of *C. albicans*

Tyrosol production was investigated by an HPLC method using planktonic cells of four *C. albicans* strains: *C. albicans* SC5314 (wild type) and the morphological mutants JKC19 (*cph1/cph1*), HLC52 (*efg1/efg1*) and HLC54 (*cph1/cph1 efg1/efg1*). Tyrosol production was determined over 48h. Tyrosol was not detectable at 5h but it was present at 10h in culture supernatants of all four *C. albicans* strains at concentrations ranging from of 0.09 to 1.28 μM (Fig. 37). After 10h, however, tyrosol production increased rapidly in all strains and reached concentrations of more than 11.6 ±1.3 μM at 48h (mean ± SEM).

3.5 Tyrosol production by biofilms of *C. albicans*

Biofilms in this experiment were grown in 75-cm^2 polystyrene tissue culture flasks to produce a sufficient volume of supernatant (>100ml) for the HPLC assay. The results (Fig. 38) show that tyrosol was detectable at 5h (0.07, 0.1 and 0.1 μM for *C. albicans* SC5314, HLC52 and HLC54, respectively). At 10h of incubation, tyrosol production was 1.15 to 1.35 μM for biofilms of all strains. From 10 to 48h, tyrosol production increased rapidly and reached concentrations of 9.3 ± 0.7 to 13.6 ± 3.5 μM (Fig. 38). Moreover, there was good correlation between tyrosol production and cell dry weight in nearly all *C. albicans* strains grown planktonically (Fig. 37) or as biofilms (Fig 38). These results support the notion that tyrosol acts as a quorum sensing molecule in *C. albicans*.

3.6 Comparison of tyrosol production by planktonic cells and biofilms on the basis of cell dry weight

To compare tyrosol production in biofilms and planktonic cells of these strains, tyrosol production was calculated in relation to cell dry weight (Fig. 39). It became clear that biofilm cells produce significantly more tyrosol than do planktonic cells. For example, tyrosol production at 48h by biofilms of *C. albicans* SC5314 and JKC 19 was 9.6 ± 0.5 and 9.7 ± 0.3 nmol/mg dry wt, respectively, while for planktonic cells it was 5.7 ± 1.1 and 6.3 ± 1.5 nmol/mg dry wt ($P<0.05$; Fig. 39 A, B). With biofilms of *C. albicans* HLC52 and HLC54, tyrosol production was also significantly greater than that of planktonic cells (Fig. 39C and D). For example, at 24 and 48h tyrosol production by biofilms of *C. albicans* HLC52 was 10.1 ± 1.8 and 16.4 ± 3.2 nmol/mg dry wt, respectively, which is significantly more than that for planktonic cells (3.9 ± 0.6 and 6.0 ± 0.3 nmol/mg dry wt) at the same time points ($P<0.05$; Fig. 39 C).

Figure 37

Relationship between tyrosol production and planktonic cell dry weight of *C. albicans*

Planktonic cells of *C. albicans* SC5314 (A), JKC 19 (B), HLC52 (C) and HLC54 (D) were grown in YNB at 37 °C for 48h. At 0, 5, 10, 24 and 48h, 50ml of culture supernatant was used for measurement of tyrosol production (□) by the HPLC assay. Meanwhile, at identical time points culture samples were assayed in triplicate for cell dry weight (■). Results are means ± SEM of three independent experiments.

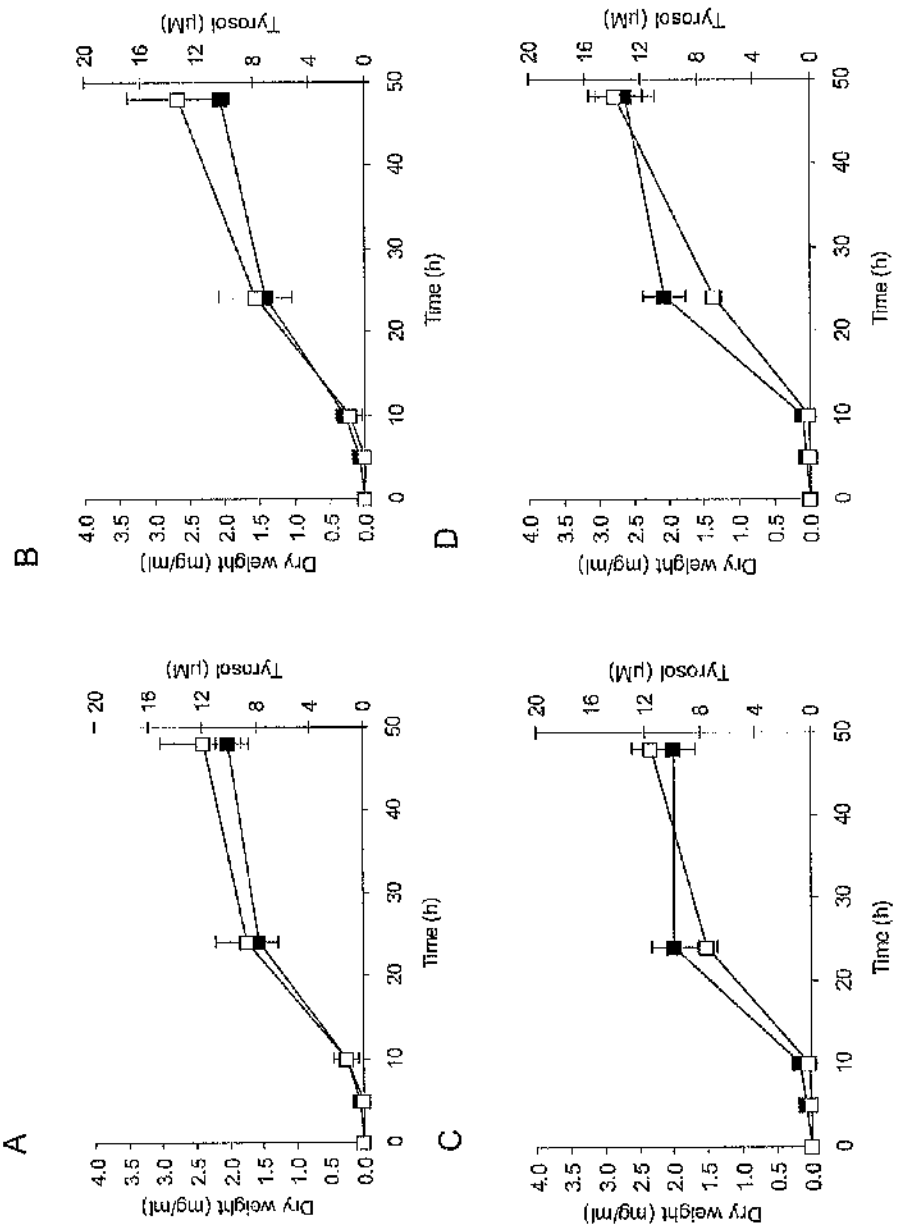

A
B
C
D
Dry weight (mg/ml)
Tyrosol (µM)
Time (h)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0
4
8
12
16
20
0
10
20
30
40
50

Figure 38

Relationship between tyrosol production and biofilm cell dry weight

Biofilms of *C. albicans* SC5314 (A), JKC 19 (B), HLC52 (C) and HLC54 (D) were grown in YNB in 75-cm^2 polystyrene tissue culture flasks at 37 °C for 48h. At 0, 5, 10, 24 and 48h, 50ml of culture supernatant was used for measurement of tyrosol production (□) by the HPLC assay. Meanwhile, at identical time points culture samples were assayed in triplicate for cell dry weight (■). Results are means ± SEM of three independent experiments.

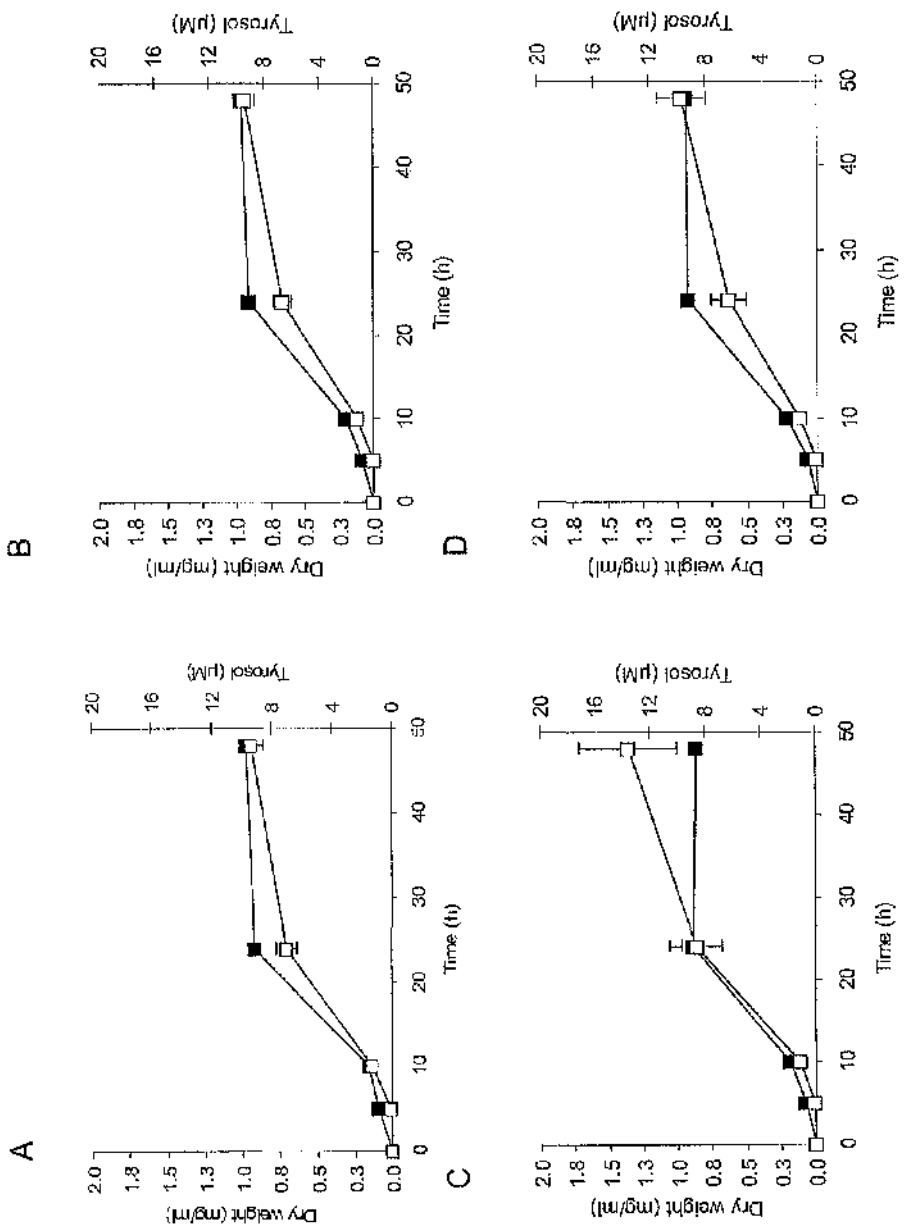
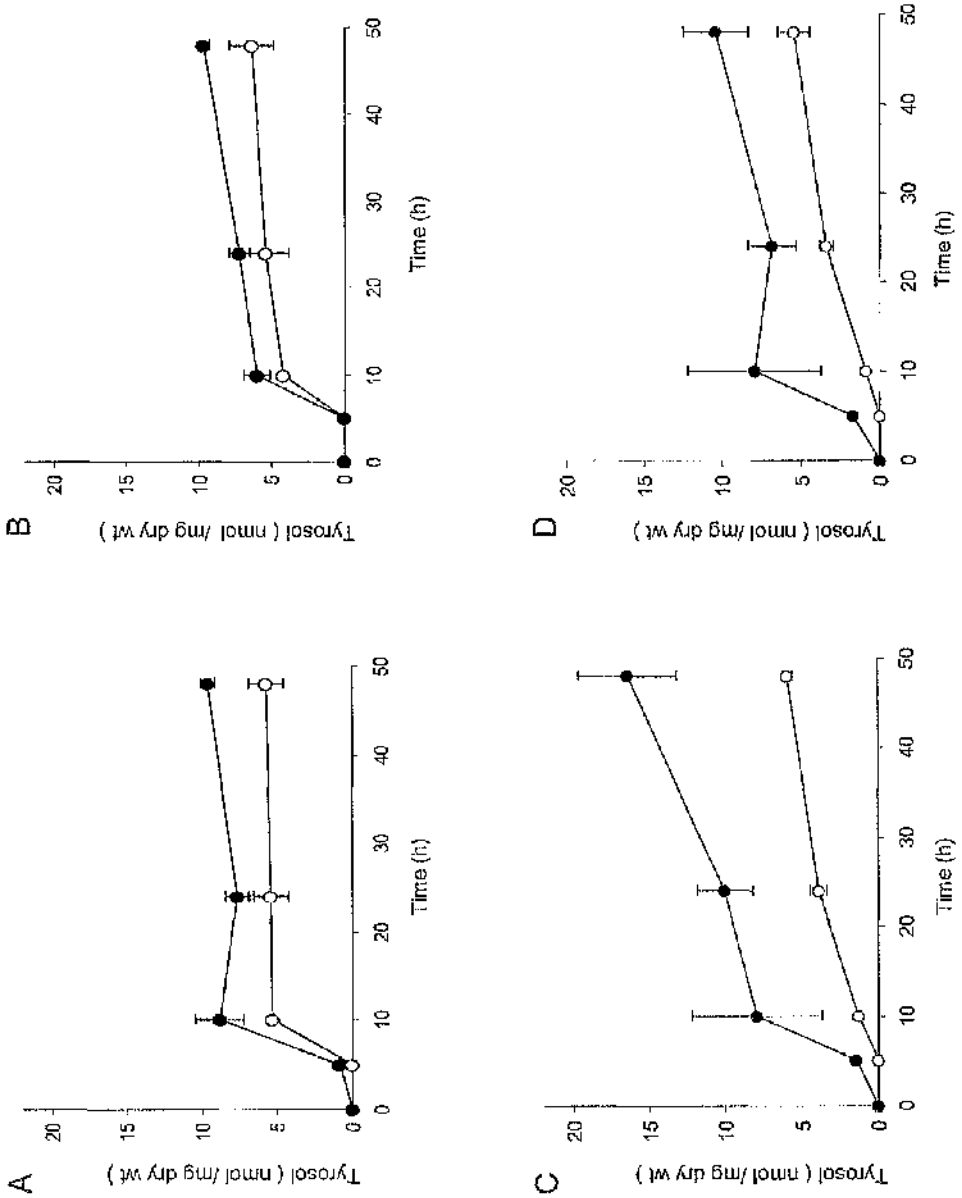

A
B
C
D
Dry weight (mg/ml)
Tyrosol (µM)
Time (h)
0.0
0.3
0.5
0.8
1.0
1.3
1.5
1.8
2.0
0
4
8
12
16
20
0
10
20
30
40
50

Figure 39

Tyrosol production expressed as a function of cell dry weight for planktonic cells and biofilms

Tyrosol production is expressed as a function of cell dry weight of *C. albicans* SC5314 (A),JKC 19 (B), HLC52 (C) and HLC54 (D) grown as planktonic cells (○) or biofilms (●). The results are means ± SEM of three independent experiments carried out in triplicate.


A
B
C
D
Tyrosol (nmol /mg dry wt)
Time (h)
0
5
10
15
20
30
40
50

3.7 Effect of simultaneous addition of farnesol and tyrosol on biofilm formation

To determine whether the effect of farnesol as a negative quorum-sensing molecule could be abolished by the effect of tyrosol as a positive quorum-sensing molecule, tyrosol at different concentrations (100, 500 and 1000 μM) was added together with farnesol at different concentrations for the adhesion period, and throughout the 48-h incubation. All tyrosol concentrations abolished the effect of 50 μM farnesol, as can be seen by comparing the results with those for 50 μM farnesol alone which inhibited biofilm formation by 15% (P<0.05; Fig. 40). The effect of 100 μM farnesol was abolished only by 1 mM tyrosol. Farnesol at 1 mM inhibited biofilm formation by 29 %, and none of the three tyrosol concentrations was able to abolish this inhibitory effect (Fig. 40).

3.8 Scanning electron microscopy of biofilm formation in the presence of farnesol and/or tyrosol

Previous studies have shown that farnesol prevents hyphal formation and biofilm development in *C. albicans* (Ramage *et al.*, 2002a), whereas conversely tyrosol promotes hyphal formation. In this study, the development of *C. albicans* biofilms in the presence of these two quorum-sensing molecules was investigated by scanning electron microscopy. Biofilms of *C. albicans* SC5314 were formed on polystyrene disks cut from 75-cm^2 tissue culture flasks (to match the procedure used for tyrosol detection by HPLC) over 48h in the presence of 1 mM farnesol and/or 500 μM tyrosol. Scanning electron microscopy showed that farnesol-

treated biofilms consisted of yeast cells only (Fig. 41 B), while control biofilms or tyrosol-treated biofilms contained a mixture of yeasts and hyphae (Fig 41, A and D). Biofilms formed in the presence of both farnesol and tyrosol on the other hand, consisted almost exclusively of yeasts (Fig. 41 C). This indicates that tyrosol did not reverse the effect of farnesol in preventing hyphal formation at these concentrations. It also demonstrates the complexity of interactions between these two quorum-sensing molecules in regulating *C. albicans* biofilm formation.

3.9 Scanning electron microscopy of the effect of tyrosol on the early stages of biofilm formation by *C. albicans*

Previous work demonstrated that tyrosol accelerates the morphological conversion (yeast cells to filaments) in *C. albicans* (Chen *et al.*, 2004). To investigate this phenomenon with biofilms, scanning electron microscopy was carried out at 1, 2, 3 and 6h of biofilm formation by *C. albicans* SC5314 in the presence or absence of 50 μM tyrosol. Tyrosol was added at time zero of biofilm formation. The results (Fig. 42 B) showed that tyrosol increases hyphal formation between at 2h and 6h of biofilm development, as compared with control biofilms incubated in the absence of tyrosol (Fig. 42 A).

Figure 40

Effect of simultaneous addition of farnesol and tyrosol on biofilm formation by *C. albicans* GDH 2346

Farnesol and tyrosol were present during the adhesion period and throughout the 48-h incubation. Biofilm formation (XTT reduction) is expressed as a percentage of that of control biofilms incubated in the absence of farnesol or tyrosol. Results are means ± SEM from two independent experiments done in duplicate. The mean (± SEM) control value for biofilm formation in the absence of farnesol (A_{492}) was 2.477 ± 0.099.

* Values significantly different from the control values at $P < 0.05$

* * Values significantly different from the control values at $P < 0.001$

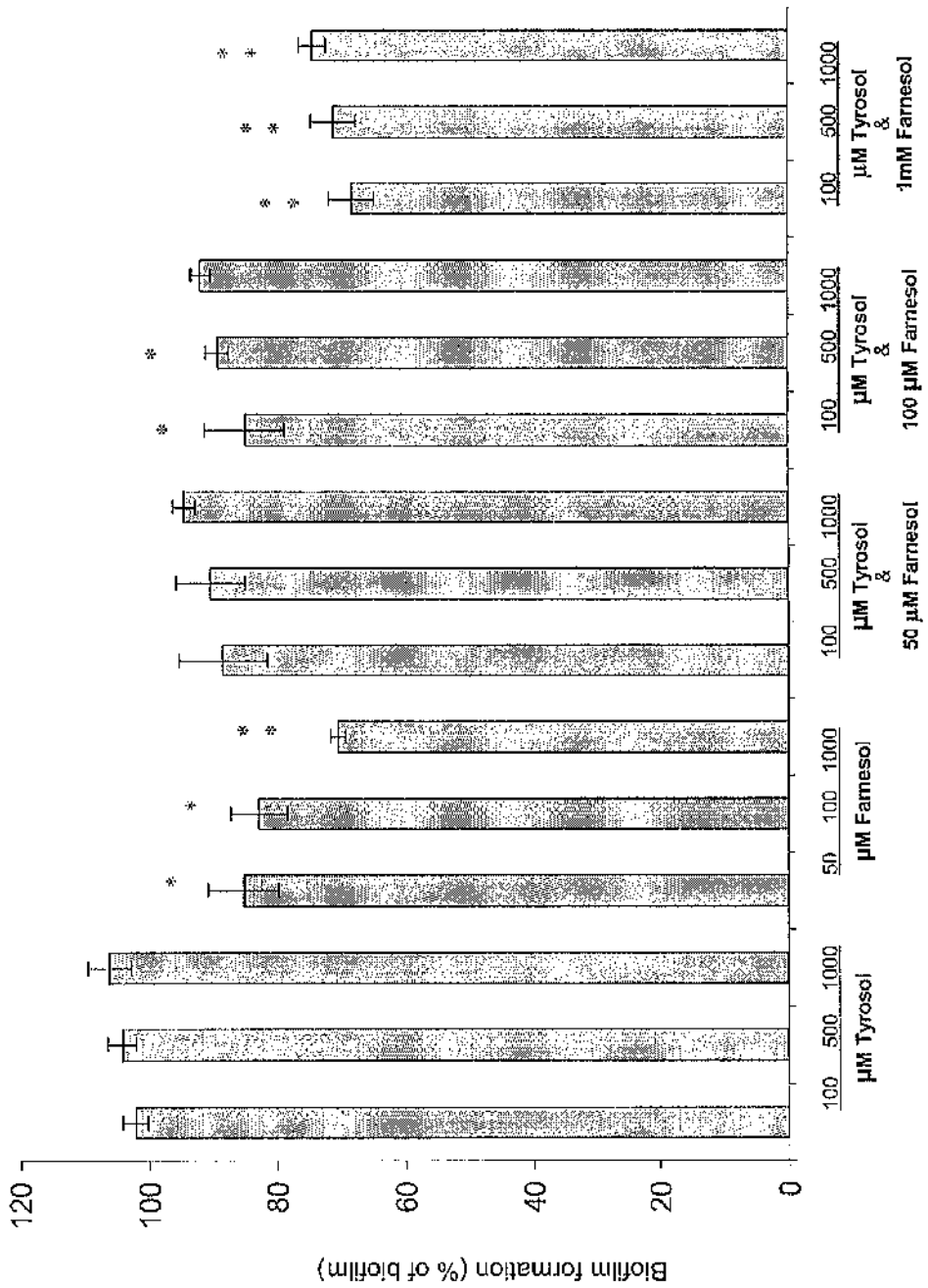

Biofilm formation (% of biofilm)
120
100
80
60
40
20
0
100 500 1000
µM Tyrosol
50 100 1000
µM Farnesol
100 500 1000
µM Tyrosol
&
50 µM Farnesol
100 500 1000
µM Tyrosol
&
100 µM Farnesol
100 500 1000
µM Tyrosol
&
1mM Farnesol

Figure 41

Scanning electron micrographs of *C. albicans* SC5314 biofilms grown on polystyrene disks in the presence of farnesol and tyrosol

Biofilms were grown for 48h in the presence of 1 mM farnesol and/or 500 μM tyrosol. (A) Control biofilm; (B) farnesol-treated biofilm; (C) farnesol+ tyrosol-treated biofilm; (D) tyrosol-treated biofilm. Bar, 10 μm.

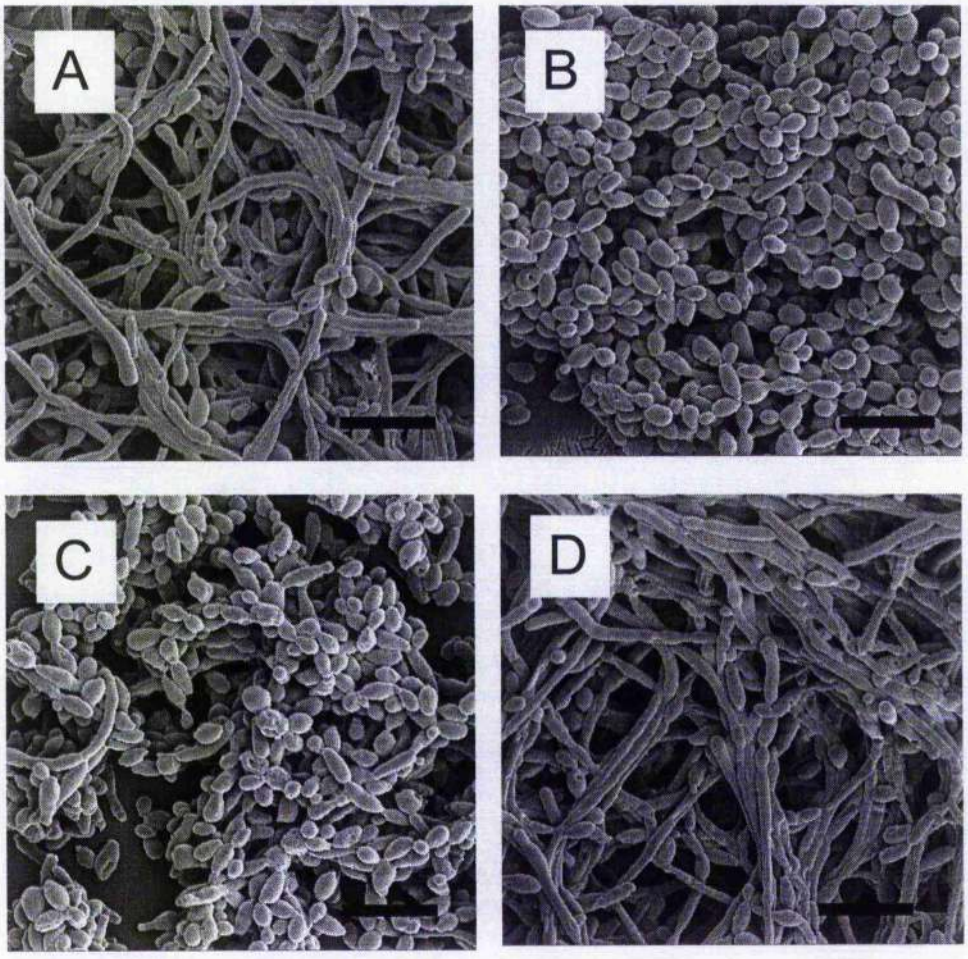
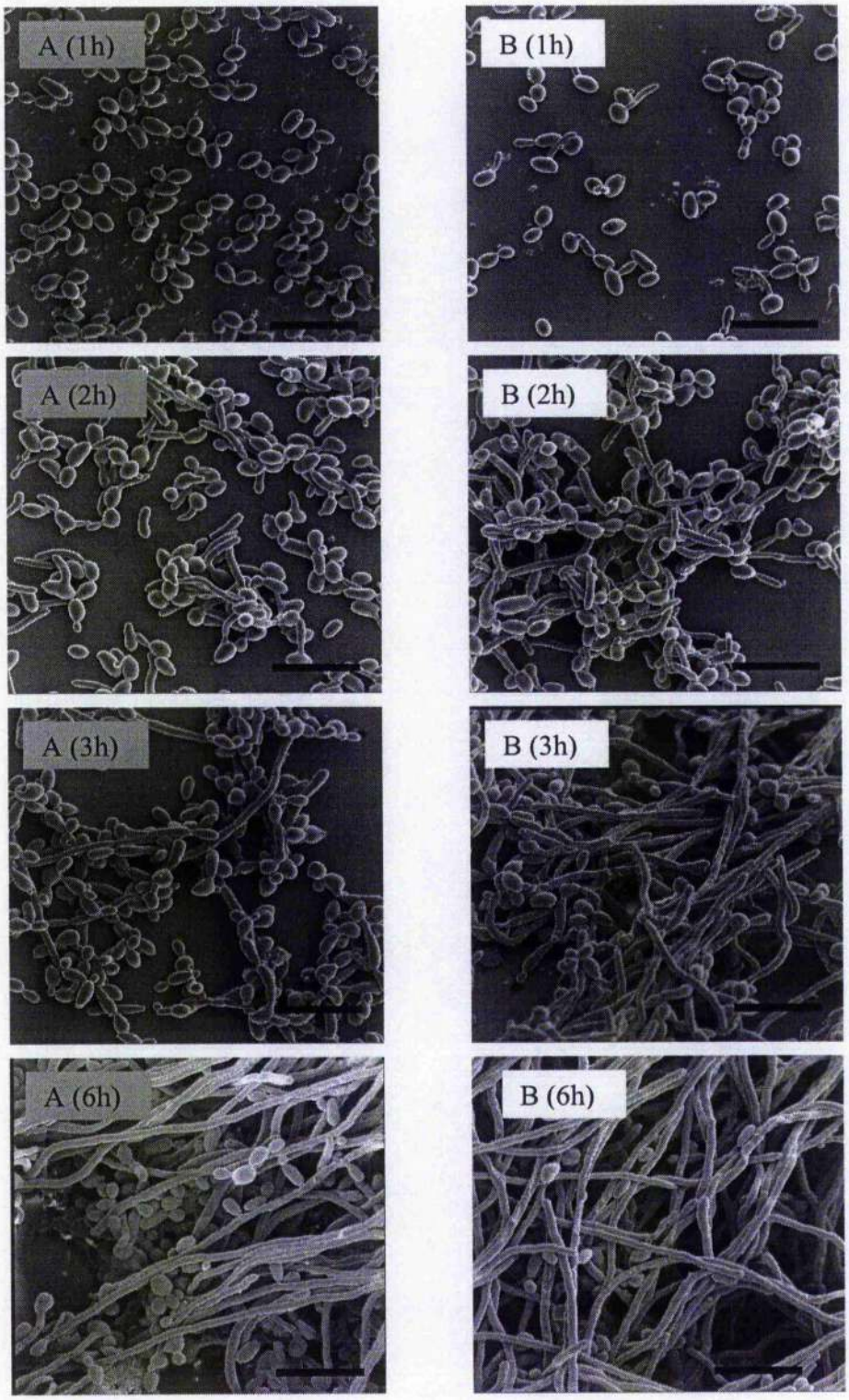

A
B
C
D

Figure 42

Scanning electron micrographs showing the effect of 50 μM tyrosol on the early stages of biofilm formation by *C. albicans*

Biofilms of *C. albicans* SC 5314 were grown on PVC catheter disks in the presence or absence of 50 μM tyrosol. (A) Control biofilms incubated for 1, 2, 3 and 6h; (B) tyrosol-treated biofilms incubated for 1, 2, 3 and 6h. Bar, 10 μm.


A (1h)
B (1h)
A (2h)
B (2h)
A (3h)
B (3h)
A (6h)
B (6h)

4. Effects of aspirin and other nonsteroidal anti-inflammatory drugs on biofilm and planktonic cells of *C. albicans*

Prostaglandins are now known to be produced by *C. albicans* and may play an important role in fungal colonization. Their synthesis in mammalian cells is decreased by inhibitors of the cyclooxygenase (COX) isoenzymes required for prostaglandin formation. In this study, a catheter disk model system was used to investigate the effect of nonsteroidal anti-inflammatory drugs, which are COX inhibitors, on biofilm formation.

4.1 Effect of PGE_2 on biofilm formation by *C. albicans*

Earlier work showed that purified fungal prostaglandin, isolated from *C. albicans*, enhanced germ-tube formation by this organism when added to incubation mixtures (Noverr *et al.*, 2001). Commercially available PGE_2 also enhanced germ-tube formation, although to a lesser extent (Kalo-Klein and Witkin, 1990; Noverr *et al.*, 2001). This finding was confirmed in the present investigation. Germ-tube formation by *C. albicans* GDH 2346 and *C. albicans* SC5314 was increased by more than 80 % over 2 h in the presence of PGE_2 (10 nM, 100 nM or 1 μM) and two different germ tube inducers (10mM proline+ 2.5mM N-acetylglucosamine or 5% serum) (Table 5). Moreover, biofilm formation by *C. albicans* GDH 2346 was increased slightly when PGE_2 was added. For example, in the presence of 100 nM PGE_2, biofilm formation was 114.8 % ± 2.4 % (mean ± SEM; $P < 0.05$) of that of control biofilms incubated in the absence of PGE_2 (Table 6).

4.2 Effect of COX inhibitors on biofilm formation

A variety of COX inhibitors were tested in biofilm assays with three strains of *C. albicans*. The compounds were all used at a final concentration of 1 mM, and were present during the adhesion period of the assay and throughout the subsequent 48-h incubation. Aspirin, etodolac (a COX-2 inhibitor) and diclofenac produced the greatest effects, with aspirin inhibiting biofilm formation by up to 95 % (Table 7), Celecoxib, nimesulide, ibuprofen and meloxicam all inhibited biofilm formation to a lesser, but still significant, extent. Indomethacin and piroxicam failed to produce significant inhibition with any of the strains tested (Table 7). Similar results for aspirin were obtained with a number of other *C. albicans* strains; 1 mM aspirin again inhibited biofilm formation by more than 88% (Fig 43). Salicylic acid which, like aspirin, has been a known NSAID for over a century, also significantly inhibited biofilm formation by more than 75 % ($P<0.001$) at concentrations of 0.1 to 1 mM (Table 8). Salicylate has virtually no activity against purified COX-1 or COX-2, and until recently its mechanism of action was not understood. It is now thought to inhibit prostaglandin synthesis in intact cells by suppressing COX-2 gene transcription (Xu *et al.*, 1999).

Table 5. Effect of PGE_2 on germ-tube formation by two *C. albicans* strains

PGE_2 concentration	Germ-tube formation (%)			
	C. albicans GDH 2346		*C. albicans* SC5314	
	Pro+GlcNAc	5% serum	Pro+GlcNAc	5% serum
1μM	46.3± 3.2[b]	31.7 ± 3.8[c]	36.0 ± 7.1[c]	29.0 ± 5.3[c]
100nM	45.0 ± 2.9[b]	32.7± 4.8[c]	38.7± 2.2[a]	34.3 ± 3.2[b]
1nM	48.7± 4.7[b]	35.7± 2.0[a]	38.3 ± 8.4[c]	32.0 ± 3.1[b]
Control	24.7 ± 3.0	17.3 ± 1.2	18.3 ± 1.8	16.0 ± 1.7

Germ-tube formation for 2h at 37 °C was assayed in the presence of two different inducers: (a) 10 mM proline+2.5mM N-acetylglucosamine and (b) 5% serum, both in 50mM potassium phosphate. Germ-tube formation is expressed as the number of germ tubes counted / 100 cells. Control cells were incubated for 2 h in the absence of PGE_2. Results are means ± SEM of two independent experiments done in triplicate.

[a] Value significantly different at $P < 0.001$ from that of control.

[b] Value significantly different at $P < 0.01$ from that of control.

[c] Value significantly different at $P < 0.05$ for that of control.

Table 6. Biofilm formation by *C. albicans* GDH 2346 in the presence of PGE_2

Prostaglandin concentration	XTT reduction (%) by biofilms after 48 h
10nM	112.9 ± 2.4
100nM	114.8 ± 2.4[a]
1 μM	115.3 ± 2.5[a]

PGE_2 was present during the adhesion period and throughout the 48-h growth period. XTT reduction is expressed as a percentage of that of control biofilms of the same strain incubated in the absence of PGE_2. Results are means ± SEM from two independent experiments carried out in triplicate. The mean (± SEM) control value (A_{492}) was 2.498 ± 0.100.

[a] Value significantly different at $P < 0.05$ for that of control.

Table 7. Effects of COX inhibitors on biofilm formation by three strains of *C. albicans*

Inhibitor	XTT reduction (%) [a] by biofilms after 48 h		
	Strain GDH 2346	Strain SC5314	Strain CAI4
Aspirin	20.6 ± 2.8 [b]	12.3 ± 2.2 [b]	4.6 ± 0.8 [b]
Diclofenac	57.6 ± 3.6 [b]	31.2 ± 1.1 [b]	59.7 ± 2.6 [b]
Ibuprofen	82.7 ± 3.9 [c]	81.7 ± 6.3 [c]	88.4 ± 6.3 [d]
Indomethacin	92.7 ± 2.8	95.8 ± 4.2	85.7 ± 13.8
Piroxicam	93.7 ± 2.9	84.4 ± 6.0	80.6 ± 7.4
Celecoxib [e]	77.3 ± 2.7 [b]	79.5 ± 7.4 [c]	94.0 ± 3.9
Etodolac [e]	62.9 ± 1.5 [b]	49.4 ± 3.8 [b]	73.4 ± 3.6 [b]
Meloxicam [e]	88.4 ± 7.0 [d]	84.5 ± 3.0 [b]	81.3 ± 2.4 [b]
Nimesulide [e]	84.8 ± 3.5 [c]	86.7 ± 7.8 [d]	94.4 ± 6.3

[a] Inhibitors were present during the adhesion period and throughout the 48-h growth period. XTT reduction is expressed as a percentage of that of control biofilms of the same strain incubated in the absence of inhibitor. Results are means ± SEM from at least two independent experiments carried out in triplicate. Mean (± SEM) control values (A_{492}) were 2.413 ± 0.069, 2.676 ± 0.066 and 2.833. ± 0.072 for strains GDH 2346, SC5314 and CAI4, respectively.

[b] Value significantly different at $P < 0.001$ from that of control.

[c] Value significantly different at $P < 0.01$ from that of control.

[d] Value significantly different at $P < 0.05$ for that of control.

[e] COX-2 inhibitor.

Figure 43

Effect of 1 mM aspirin on biofilm formation by different *C. albicans* strains

1 mM aspirin was present during the adhesion period and throughout the 48-h growth period. Biofilm formation (XTT reduction) is expressed as a percentage of that of control biofilms of the same strain incubated in the absence of aspirin. Results are means ± SEM from at least two independent experiments carried out in triplicate. Mean (± SEM) control values (A_{492}) ranged from 2.048± 0.219 to 2.809± 0.320 for all *C. albicans* strains.

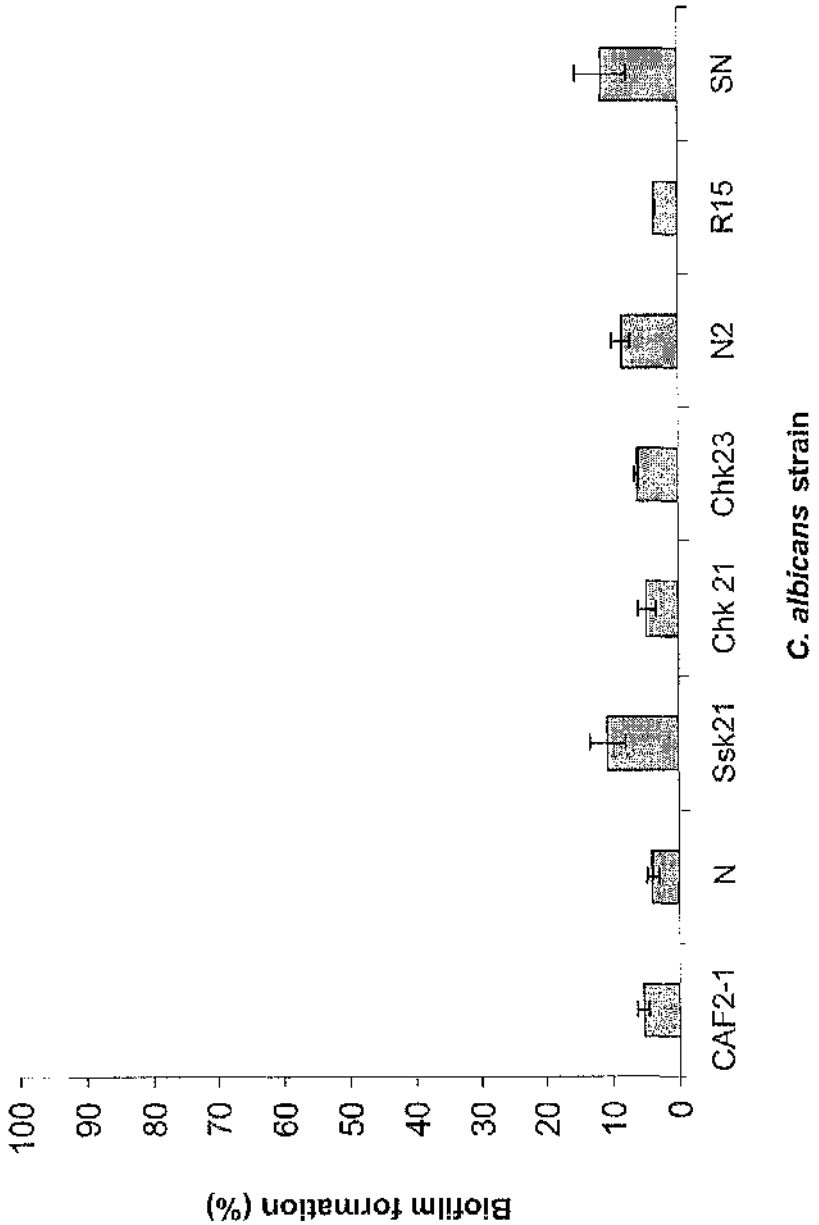

Biofilm formation (%)
100
90
80
70
60
50
40
30
20
10
0
CAF2-1
N
Ssk21
Chk 21
Chk23
N2
R15
SN
C. albicans strain

Table 8. Effect of salicylic acid on biofilm formation by three strains of *C. albicans*

Salicylic acid concentration	XTT reduction (%) [a] by biofilms after 48 h		
	Strain GDH 2346	Strain SC5314	Strain CAI4
100 μM	26.1 ± 5.4	11.0 ± 1.5	21.7 ± 2.0
500 μM	16.5 ± 1.6	7.3 ± 0.4	4.0 ± 0.4
1 mM	11.2 ± 1.8	2.8 ± 0.8	4.2 ± 1.3

[a] Salicylic acid was present during the adhesion period and throughout the 48-h growth period. XTT reduction is expressed as a percentage of that of control biofilms of the same strain incubated in the absence of inhibitor. Results are means ± SEM from two independent experiments carried out in quadruplicate. Mean (± SEM) control values (A_{492}) were 2.481 ± 0.126, 2.754 ± 0.011 and 2.655 ± 0.312 for strains GDH 2346, SC5314 and CAI4, respectively.

All results were significantly different at $P < 0.001$ from that of the control.

4.3 Inhibition of biofilm formation by different concentrations of aspirin

Although aspirin has been reported to have antifungal activity (Deva *et al.*, 2001), its dramatic inhibition of biofilm formation was unexpected, and was therefore investigated further at drug concentrations lower than 1 mM. The inhibitory effect on biofilms appeared to be dose related. Over 70 % inhibition was observed at aspirin concentrations between 100 μM and 1 mM (Fig. 44). Lower concentrations (50-75 μM) produced only around 20 % inhibition, while 10 μM aspirin had no effect on biofilm formation. Aspirin concentrations of 50-200 μM can be achieved by therapeutic doses of the drug in humans (Xu *et al.*, 1999; Wu, 2000), suggesting that the antibiofilm effect observed *in vitro* might also be relevant *in vivo*.

4.4 Aspirin addition during different stages of biofilm growth

The results presented in Table 7, Fig. 43 and Fig. 44 could conceivably be explained by the antifungal activity of aspirin on planktonic (free-floating) cells used as the inoculum for biofilm formation. To discount this possibility, the effect of adding aspirin at different times during biofilm growth was investigated. After the 1-h adhesion period, all remaining planktonic cells were removed from the disks by washing. Disks with firmly attached cells were then submerged in fresh growth medium and aspirin was added at 0, 2, 4, 24 or 48 h of the incubation period, at a final concentration of 1 mM. Biofilm formation, as determined by the metabolic activity of biofilm cells in the XTT reduction assay, was measured after 48 h (Table 9). In one experiment (Expt B), aspirin was also present during the 1-h adhesion period. Biofilm growth was severely inhibited by aspirin addition at

any time up to 24 h. For example, addition of aspirin to relatively mature, 24-h biofilms reduced their metabolic activity at 48 h by over 80% (Table 9). Drug addition at 48 h produced little or no effect because at this stage in the experiment, the biofilms were immediately harvested, washed and tested in the XTT assay. However, the 48-h result (Table 9, Expt B) does indicate that the presence of aspirin during the adhesion period had only a minimal effect on biofilm formation. Overall, these findings demonstrate that the antibiofilm effect of aspirin is not simply due to an antifungal effect on the biofilm inoculum.

4.5 Aspirin addition to mature biofilms

In a further series of experiments, mature, 48-h biofilms grown in the absence of aspirin were transferred to fresh growth medium containing different concentrations of the drug, and incubated for further periods of 5 - 48 h. Most of the aspirin concentrations tested (75 μM to 1 mM) significantly inhibited biofilm metabolic activity and viability after 16 h (Tables 10 and 11). After 48 h of additional incubation, biofilm activity and viability was reduced by 17-87 %. Moreover, physiological concentrations of the drug (75-200 μM) reduced activity by 17-87%, suggesting that aspirin could have a significant inhibitory effect on mature biofilms *in vivo*.

Figure 44

Inhibition of biofilm formation by *C. albicans* GDH 2346 by different concentrations of aspirin

Biofilm formation (as measured by XTT reduction) is expressed as a percentage of that of control biofilms incubated in the absence of aspirin. Most results are means ± SEM from at least two independent experiments with a total of seven or more replicates. Results for 250 μM and 500 μM aspirin are means ± SEM of triplicate determinations. The mean (± SEM) control value (A492) was 2.212 ± 0.084.

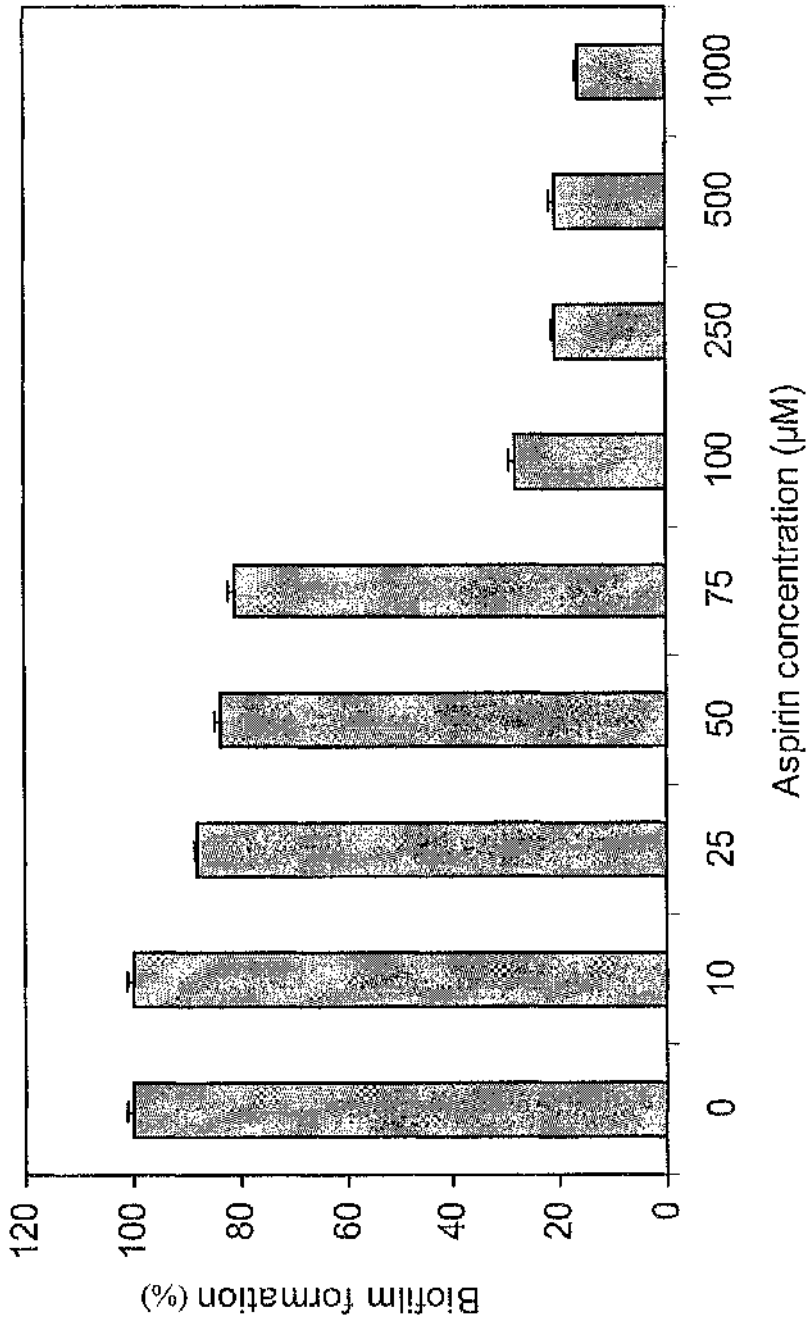

Biofilm formation (%)
120
100
80
60
40
20
0
0
10
25
50
75
100
250
500
1000
Aspirin concentration (μM)

Table 9. Effect of aspirin addition at different times during biofilm formation by *C. albicans* SC5314

Time of aspirin (1 mM) addition (h)	XTT reduction (%) [a] by biofilms at 48 h	
	Expt A	Expt B
0	10.8 ± 0.8 [b]	8.0 ± 1.8 [b]
2	13.4 ± 1.2 [b]	7.6 ± 0.6 [b]
4	18.7 ± 2.4 [b]	8.3 ± 0.7 [b]
24	18.8 ± 4.7 [b]	12.1 ± 1.5 [b]
48	103.6 ± 4.0	94.3 ± 3.4

[a] XTT reduction is expressed as a percentage of that of control biofilms incubated for 48 h in the absence of aspirin. Results are means ± SEM of four replicate determinations. In Expt B, aspirin was initially present at a concentration of 1 mM during the 1-h adhesion period of all assays, after which it was removed along with unattached yeasts during washing. The drug was then added at the times indicated during the subsequent incubation period. In Expt A, no aspirin was present during the adhesion period. Mean (± SEM) control values (A_{492}) were 2.532 ± 0.081 (Expt A) and 2.979 ± 0.122 (Expt B).

[b] Value significantly different at $P < 0.001$ from that of control.

Table 10. Effect of aspirin on XTT reduction by mature (48-h) biofilms of *C. albicans* GDH 2346

Period of further incubation (h)	XTT reduction (%) [a] after further incubation in medium containing aspirin at a concentration of			
	75 μM	100 μM	200 μM	1 mM
5	100.8 ± 2.7	99.8 ± 2.7	79.3 ± 3.3 [b]	66.0 ± 2.6 [b]
16	89.7 ± 4.1 [d]	87.8 ± 3.2 [c]	80.1 ± 4.0 [b]	58.2 ± 3.0 [b]
24	94.4 ± 5.2	85.6 ± 4.1 [c]	78.3 ± 2.6 [b]	41.8 ± 2.0 [b]
48	80.8 ± 6.5 [c]	44.4 ± 2.7 [b]	20.2 ± 1.6 [b]	10.3 ± 1.1 [b]

[a] Different concentrations of aspirin were added to mature (48-h) biofilms grown in the absence of the drug, and incubation was continued for a further 5 - 48 h. XTT reduction is expressed as a percentage of that of control 48-h biofilms incubated for the same time period without aspirin. Results are means ± SEM for two independent experiments carried out in quadruplicate. Mean (± SEM) control values (A_{492}) for different incubation periods ranged from 2.556 ± 0.033 to 2.807 ± 0.080.

[b] Value significantly different at $P < 0.001$ from that of control.

[c] Value significantly different at $P < 0.01$ from that of control.

[d] Value significantly different at $P < 0.05$ for that of control.

Table 11. Effect of aspirin on viability of mature (48-h) biofilms of *C. albicans* GDH 2346

Period of further incubation (h)	Viability (%) [a] after further incubation in medium containing aspirin at a concentration of			
	75 μM	100 μM	200 μM	1 mM
5	97.6 ± 6.4	89.4 ± 2.3	87.6 ± 6.0	81.9 ± 3.3 [d]
16	90.0 ± 8.8	84.9 ± 3.0 [c]	77.2 ± 6.6 [d]	65.7 ± 2.2 [c]
24	87.5 ± 15.9	69.3 ± 3.5 [c]	70.2 ± 2.5 [b]	50.6 ± 4.1 [b]
48	83.3 ± 11.4	30.3 ± 2.5 [b]	13.0 ± 0.4 [b]	4.7 ± 0.2 [b]

[a] Different concentrations of aspirin were added to mature (48-h) biofilms grown in the absence of the drug, and incubation was continued for a further 5 - 48 h. Viability is expressed as a percentage of that of control cells incubated under identical conditions in the absence of aspirin. Results are means ± SEM of at least four replicate determinations. Controls counts gave mean values of $4.03 \times 10^6 \pm 2.8 \times 10^3$ to $6.00 \times 10^6 \pm 2.3 \times 10^3$ cells / biofilm disk. Viable counts were carried out by the standard procedure of serial dilution followed by plating. Cells from triplicate disks were resuspended in 3 ml of PBS and were then diluted and plated.

[b] Value significantly different at $P < 0.001$ from that of control.

[c] Value significantly different at $P < 0.01$ from that of control.

[d] Value significantly different at $P < 0.05$ for that of control.

4.6 Effect of simultaneous addition of aspirin and PGE_2 on biofilm formation

To investigate whether the antibiofilm effect of aspirin might be related to an inhibition of fungal prostaglandin production, PGE_2 was added simultaneously with aspirin at the beginning of the 1-h adhesion period, and again at time zero of the subsequent 48-h incubation. Aspirin alone, at a concentration of either 25 μM or 50 μM, significantly inhibited biofilm formation (Table 12). However, 1 nM or100 nM PGE_2 completely abolished the inhibition due to aspirin at these relatively low concentrations, indicating a possible role for prostaglandin(s) in the regulation of biofilm formation.

4.7 Viability of biofilms after treatment with COX inhibitors

Viable counts were carried out on both biofilm and planktonic cells of *C. albicans* GDH 2346 after growth for 24 h in the presence of 1 mM aspirin, etodolac, indomethacin or piroxicam. Aspirin, whose antifungal properties have been reported previously (Deva *et al.*, 2000), drastically reduced the viability of planktonic cells to 1.0 % of that of untreated control cells (Table 13). Surprisingly, however, it also reduced the viability of normally recalcitrant biofilm cells to 1.9 % of that of untreated controls. A longer incubation period with aspirin (48 h) decreased viability still further to 0.1 % of that of controls for both cell types (data not shown). With the other COX inhibitors, viability in most cases was reduced to around 40% after 24 h although, interestingly, biofilm cells were completely resistant to indomethacin.

Table 12. Effect of simultaneous addition of aspirin and PGE_2 on biofilm formation by *C. albicans* GDH 2346

Addition	XTT reduction (%) [a] by biofilms at 48 h
Aspirin (25μM)	87.7 ± 9.5 [c]
+ PGE_2 (1 nM)	106.1 ± 6.0
+ PGE_2 (100 nM)	101.3 ± 8.9
Aspirin (50 μM)	79.0 ± 7.6 [b]
+ PGE_2 (1 nM)	101.2 ± 11.3
+ PGE_2 (100 nM)	102.9 ± 7.8

[a] XTT reduction is expressed as a percentage of that of control 48-h biofilms incubated in the absence of aspirin and PGE_2. Results are means ± SEM from two independent experiments carried out with a total of seven replicates. The mean (± SEM) control value (A_{492}) was 2.500 ± 0.112.

[b] Value significantly different at $P < 0.001$ from that of control.

[c] Value significantly different at $P < 0.01$ from that of control.

Table 13. Viability of planktonic and biofilm cells of *C. albicans* GDH 2346 after growth for 24 h in the presence of different COX inhibitors [a]

Inhibitor (1 mM)	Viability (%)	
	Planktonic cells	Biofilm cells
Etodolac	39.2 ± 1.7	38.7 ± 2.3
Indomethacin	41.6 ± 0.7	98.7 ± 1.0
Piroxicam	40.6 ± 3.1	37.9 ± 2.6
Aspirin	1.0 ± 0.1	1.9 ± 0.1
Salicylic acid	4.9 ± 0.1	0.8 ± 0.1

[a] Viability is expressed as a percentage of that of control cells incubated under identical conditions in the absence of inhibitor. Results are means ± SEM of at least four replicate determinations. Control counts gave mean values of $14.7 \times 10^6 \pm 1.5 \times 10^6$ cells / ml for planktonic cells and $1.9 \times 10^6 \pm 0.1 \times 10^6$ to $10.4 \times 10^6 \pm 0.6 \times 10^6$ cells / disk for biofilms. Viable counts were carried out by the standard procedure of serial dilution followed by plating. For biofilm counts, cells from triplicate disks were resuspended in 3 ml of PBS and were then diluted and plated.

4.8 Scanning electron microscopy of biofilms exposed to COX inhibitors

Morphogenesis appears to be an important feature of the development of *C. albicans* biofilms under many environmental conditions (Douglas, 2003). Biofilms grown on catheter disks normally consist of two distinct layers: a thin, basal region of densely packed yeast cells and an overlying, mainly hyphal layer (Baillie and Douglas, 1999b). In this study, the morphological appearance of biofilms of *C. albicans* GDH 2346 was examined by scanning electron microscopy after growth in the presence of various COX inhibitors. Aspirin reduced biofilm formation substantially as determined by quantitative measurements (Table 7 and Fig 43), but in areas of the catheter disks where biofilm could be observed, large numbers of yeasts and hyphae were present, just as in untreated controls (Fig. 45A, B). However, examination of the cells at higher magnification revealed that aspirin-treated fungi had very wrinkled surfaces (Fig. 45C, D). Piroxicam affected the appearance of biofilms very little (Fig. 45B), whereas treatment with either indomethacin or etodolac resulted in biofilms that consisted almost entirely of yeast cells (Fig. 45C, D). The change in morphology due to indomethacin is particularly noteworthy, since this drug affected neither cell viability (Table 13) nor the extent of biofilm formation, as determined by XTT assays (Table 7 and Fig. 43).

4.9 Effect of COX inhibitors on germ-tube formation

Germ-tube formation by planktonic *C. albicans* GDH 2346 in the presence of COX inhibitors (100 μM) was determined in proline/N-acetylglucosamine buffer over 2 h. Indomethacin had the greatest effect, inhibiting germ-tube

formation by almost 80% (Fig. 47). This is consistent with the absence of hyphae in developing biofilms exposed to the drug (Fig. 46C). Treatment with celecoxib or ibuprofen also resulted in significant inhibition (>70%; Fig. 47). The compound that inhibited germ-tube formation least was aspirin, a finding that correlated with the presence of both yeasts and hyphae in biofilms exposed to this drug (Fig. 45B).

4.10 Effect of simultaneous addition of aspirin and cAMP on biofilm formation by *C. albicans* GDH 2346

It has been reported that a cyclic adenosine monophosphate (cAMP) signalling pathway can regulate morphological changes between yeast and filamentous forms in many fungi, including *C. albicans* (Lengeler *et al.*, 2000). In this experiment, cAMP was added together with aspirin to determine whether the antibiofilm activity of aspirin could be influenced by the presence of cAMP. Table 14 shows that cAMP alone did not affect *C. albicans* biofilm formation; in contrast, 100 μM aspirin inhibited *C. albicans* biofilm formation by more than 78% in the presence or absence of cAMP ($P<0.001$). This result demonstrates that aspirin activity against biofilm formation by *C. albicans* is unlikely to involve the cAMP signalling pathway, which may explain why aspirin-treated biofilms consist of both yeast cells and hyphae (Fig. 45).

Figure 45

Scanning electron micrographs of *C. albicans* GDH 2346 biofilms grown in the presence of aspirin

Biofilms were grown on PVC catheter disks for 48 h in the presence of 1 mM aspirin. (A) Control biofilm; (B) aspirin-treated biofilm; (C) enlargement of marked square in A; (D) enlargement of marked square in B. Arrows indicate smooth surface of control cells (C) and wrinkled surface of aspirin-treated cells (D). Bars, 8μm (A, B) and 1μm (C, D).

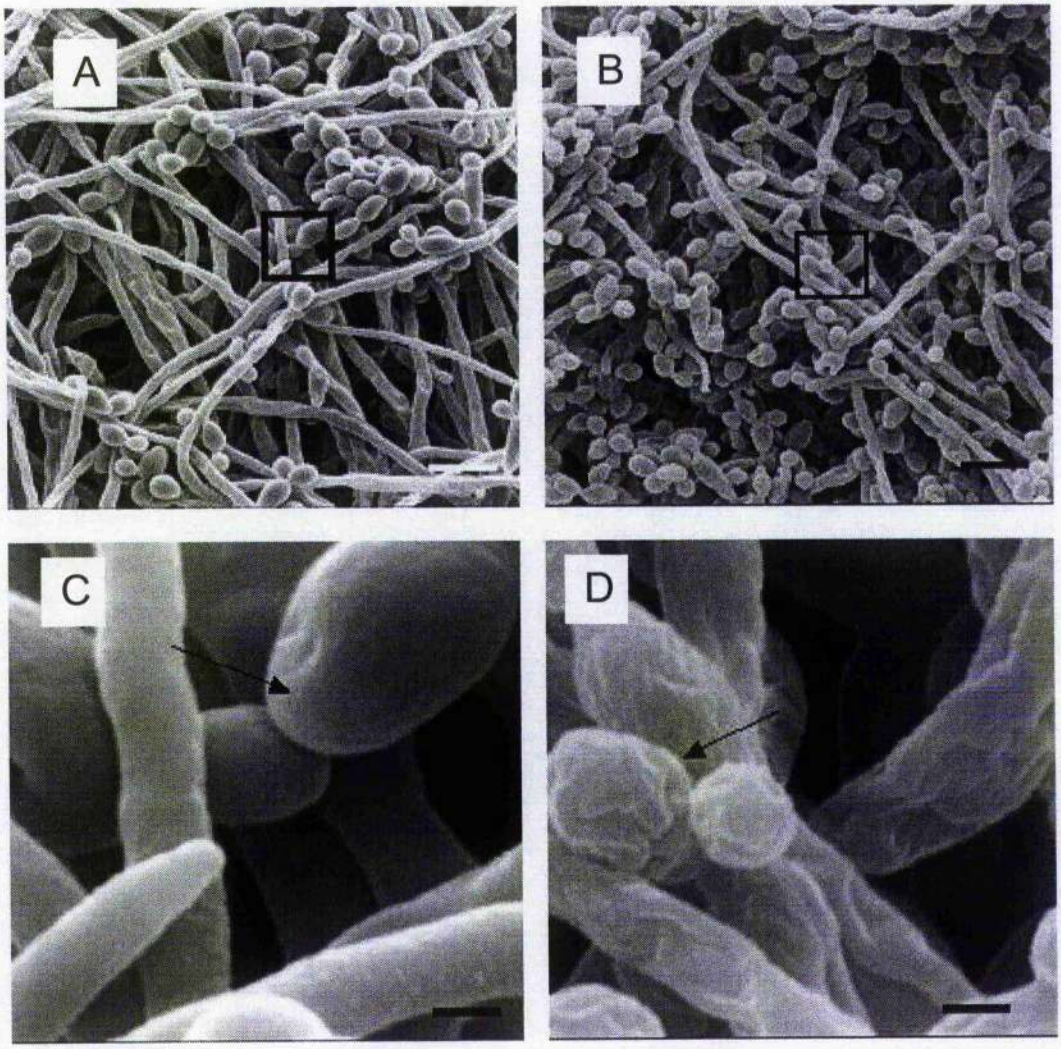

A
B
C
D

Figure 46

Scanning electron micrographs of *C. albicans* GDH 2346 biofilms grown in the presence of different COX inhibitors

Biofilms were grown on PVC catheter disks for 48 h in the presence of no inhibitor (A), 1 mM piroxicam (B), 1 mM indomethacin (C), or 1 mM etodolac (D). Bar, 8μm.

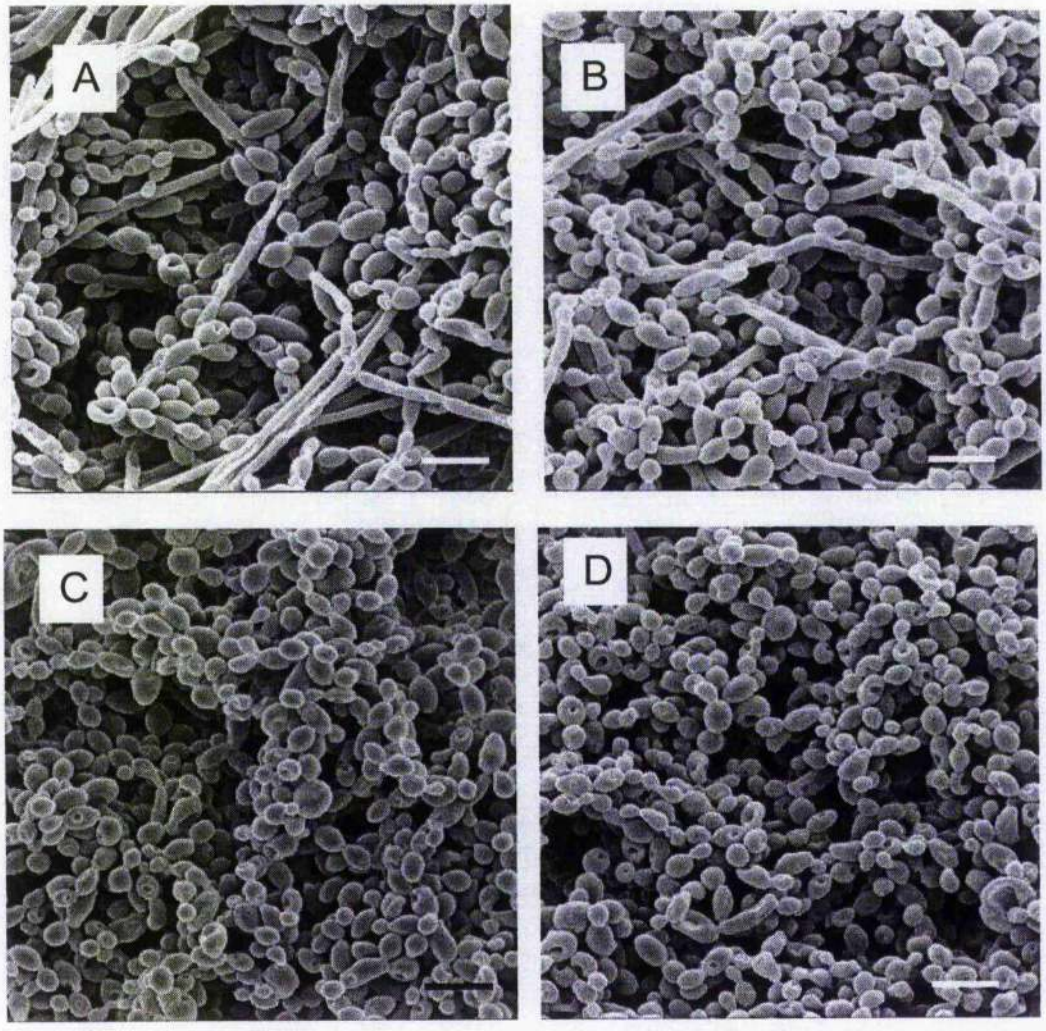

A
B
C
D

Figure 47

Effects of COX inhibitors (final concentration 100 μM) on germ-tube formation by *C. albicans* GDH 2346

Germ-tube formation is expressed as a percentage of that for control cells incubated in the absence of inhibitors. Results are means ± SEM of triplicate determinations. The mean (± SEM) control value was 72.6 ± 12 germ tube-forming cells / 200 cells counted.

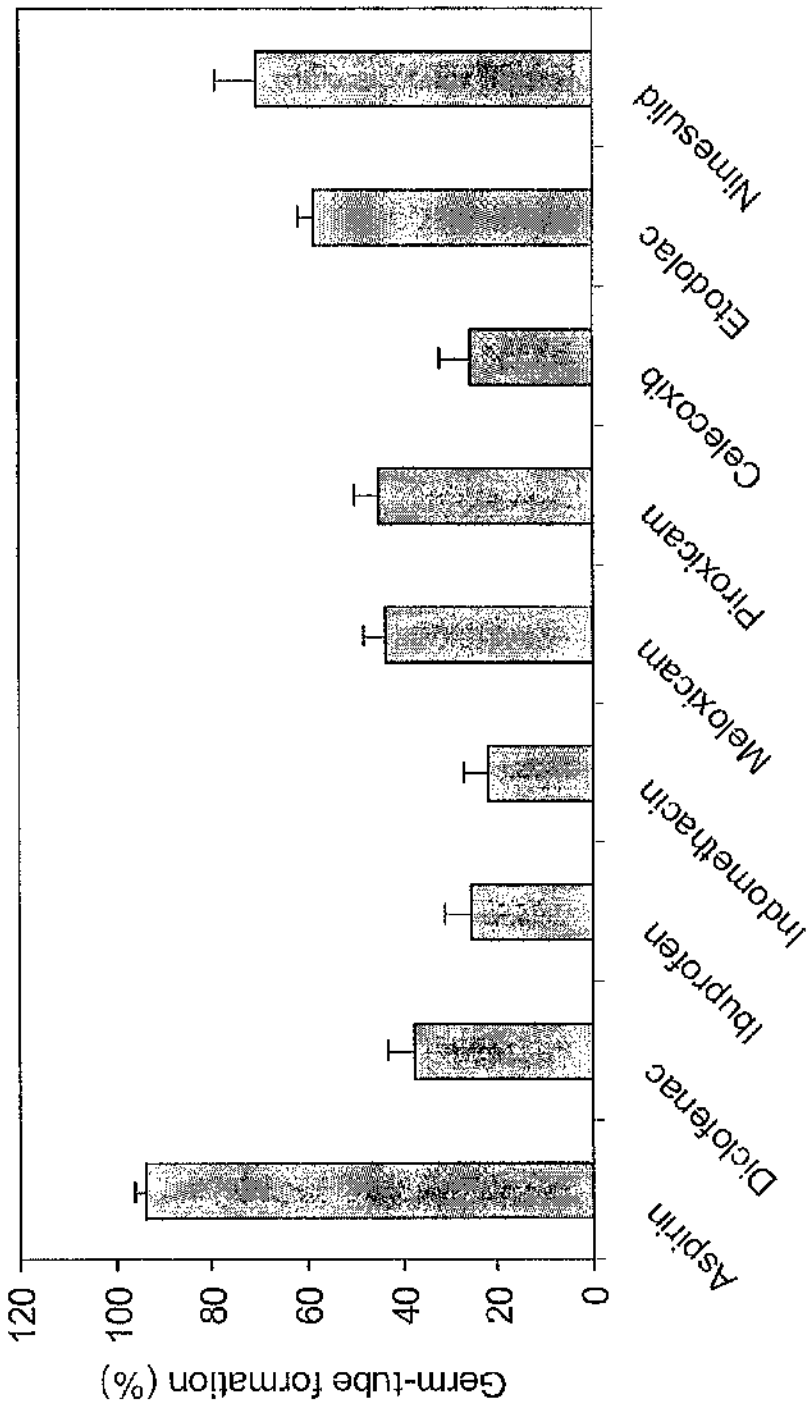

Germ-tube formation (%)
120
100
80
60
40
20
0
Aspirin
Diclofenac
Ibuprofen
Indomethacin
Meloxicam
Piroxicam
Celecoxib
Etodolac
Nimesulid

Table 14. Effect of simultaneous addition of aspirin and cAMP on biofilm formation by *C. albicans* GDH 2346

Addition	XTT reduction (%) [a] by biofilms at 48 h
cAMP (10 μM)	95.3 ± 3.0
cAMP (100 μM)	98.8 ± 3.9
Aspirin (100 μM)	21.3 ± 1.0 [b]
+ cAMP (10 μM)	18.6 ± 4.4 [b]
+ cAMP (100μM)	20.9 ± 2.2 [b]

[a] XTT reduction is expressed as a percentage of that of control 48-h biofilms incubated in the absence of aspirin and cAMP. Results are means ± SEM from at least four replicates. The mean (± SEM) control value (A_{492}) was 2.417 ± 0.304.

[b] Value significantly different at $P < 0.001$ from that of control.

5. Combined effects of COX inhibitors and antifungal agents on *Candida* biofilms

5.1 MICs of aspirin and / or amphotericin B for planktonic *C. albicans* GDH 2346

Previous work showed that a combination of fluconazole with either sodium salicylate or ibuprofen results in synergistic activity against *C. albicans* (Scott *et al.*, 1995; Pina-Vaz *et al.*, 2000). Therefore it was of interest to investigate the combined activity of COX inhibitors and antifungal agents on planktonic cell growth and biofilm formation. In these experiments, the effect of aspirin and / or amphotericin B on planktonic growth of *C. albicans* GDH 2346 was evaluated by a broth microdilution method (M27) in accordance with the recommendations of the National Committee of Clinical Laboratory Standards (NCCLS). This method was used to investigate the effects of aspirin (50μM, 75μM, 100μM and 1 mM), amphotericin B (0.0313, 0.0625, 0.125, 0.25, 0.5, 1, 2 and 4 μg/ ml) and combinations of the two drugs. Preliminary results showed that aspirin did not affect *C. albicans* cell density. On the other hand, the minimum inhibitory concentration (MIC) of amphotericin B was 0.5 μg/ml i.e. the lowest drug concentration that prevents discernible growth (data not shown). Similar results were obtained for both drugs (ie., aspirin did not affect *C. albicans* cell density and MICs of amphotericin B were 0.5 to 1.0 μg/ml) when RPMI 1640 medium was replaced in the NCCLS protocol by other media such as YNB buffered by PBS, YNB buffered by MOPS at pH 7, and unbuffered YNB (data not shown).

5.2 Effect of aspirin and amphotericin B on biofilm formation by *C. albicans* GDH 2346

Aspirin and /or amphotericin B activity against *C. albicans* biofilms grown in buffered or unbuffered YNB was evaluated. Both agents were added during the adhesion period and again at time zero of biofilm formation by *C. albicans* GDH 2346 grown for 48h in MOPS-buffered YNB (pH 7) or unbuffered YNB. The drugs were added individually or in combination and biofilm metabolic activity (XTT reduction) was measured. In unbuffered YNB, aspirin at 75 or 100 μM and amphotericin B at 0.5, 0.25 or 0.125 μg/ml significantly inhibited biofilm formation when added individually or in combination (P <0.01 to P <0.001;Table 15). In contrast, biofilm formation in buffered YNB was unaffected by the presence of aspirin or amphotericin B (Table 15).

Table 15. Effect of aspirin and amphotericin B on biofilm formation by *C. albicans* GDH 2346 in YNB (with and without MOPS)

Drug(s) added:	Biofilm formation (%) [a]	
	YNB+ MOPS	YNB
100μM aspirin	100.0 ± 1.3	34.5 ± 1.4 [b]
75μM aspirin	100.4 ± 2.8	74.5 ± 1.2 [c]
50μM aspirin	101.6 ± 2.9	86.9 ± 8.4
0.5μg/ml AmB	100.3 ± 1.4	2.1 ± 1.0 [b]
0.25 μg/ml AmB	104.1 ± 1.4	52.0 ± 1.3 [b]
0.125μg/ml AmB	102.5 ± 1.6	51.3 ± 5.5 [c]
100μM aspirin + 0.5 μg/ml AmB	92.9 ± 5.0	1.4 ± 0.6 [b]
100μM aspirin + 0.25 μg/ml AmB	94.1 ± 3.2	10.6 ± 0.8 [b]
100μM aspirin + 0.125 μg/ml AmB	106.0 ± 1.0	15.0 ± 4.6 [b]
75μM aspirin + 0.5 μg/ml AmB	98.3 ± 1.6	3.9 ± 0.2 [b]
75μM aspirin + 0.25 μg/ml AmB	101.7 ± 1.5	12.7 ± 1.7 [b]
75μM aspirin + 0.125 μg/ml AmB	100.9 ± 0.8	12.4 ± 1.7 [b]
50μM aspirin + 0.5 μg/ml AmB	96.6 ± 1.4	1.4 ± 1.2 [b]
50μM aspirin + 0.25 μg/ml AmB	103.8 ± 0.4	20.5 ± 1.3 [b]
50μM aspirin + 0.125 μg/ml AmB	102.9 ± 1.0	21.5 ± 3.0 [b]

[a] Aspirin and amphotericin B were present during the adhesion period and throughout the 48-h growth period. XTT reduction is expressed as a percentage of that of control biofilms incubated in the absence of aspirin or AmB. Results are means ± SEM from at least two independent experiments carried out in triplicate. Mean (± SEM) control values (A_{492}) were 3.135 ± 0.129 and 2.548 ± 0.121 for *C. albicans* GDH 2346 grown in YNB+MOPS and YNB respectively

[b] Value significantly different at $P < 0.001$ from that of control.

[c] Value significantly different at $P < 0.01$ from that of control.

5.3 Effect of aspirin, salicylic acid and amphotericin B on biofilm formation by *C. albicans* GDII 2346 in different media

Aspirin, as shown in the previous section, had no significant effect on *C. albicans* biofilms grown in YNB buffered with MOPS. To determine whether the buffer could influence COX inhibitor activity, aspirin, salicylic acid and amphotericin B were tested against biofilms of *C. albicans* grown in YNB, YNB buffered with PBS or MOPS, and RPMI 1640 buffered with HEPES (Table 16). The pH values of the media were measured before and after incubation (Table 16, footnote). The results show that there was a significant decrease in biofilm formation in YNB with 1 mM aspirin or 1 mM salicylic acid; inhibition was 79 % and 84 %, respectively ($P<0.001$). These inhibition effects were decreased but still significant when biofilms were grown in YNB+PBS. For example, aspirin and salicylic acid inhibited biofilm formation by 38 % and 22 %, respectively ($P<0.01$ and $P< 0.05$; Table 16). However, aspirin and salicylic acid did not inhibit biofilm formation in YNB+MOPS or RPMI 1640+HEPES. In contrast, amphotericin B at 2 or 10 µg/ml inhibited biofilm formation by more than 99% in all four media (Table 16).

5.4 Effect of COX inhibitors other than aspirin on biofilm formation by three *C. albicans* strains grown in two different media

The effect of COX inhibitors other than aspirin (etodolac, indomethacin and piroxicam) was further investigated to determine whether the pK_a (ionization constant) of these COX inhibitors could affect their activity, since the pH value of

the growth medium was higher than the pK_a value of the inhibitors. It is known that aspirin has a pK_a value of 3.5, which could explain why aspirin did not inhibit *Candida* biofilms grown in media buffered to pH 7.0. Therefore, other COX inhibitors (etodolac, pK_a 4.7; indomethacin, pK_a 4.5; and piroxicam, pK_a 6.3) were tested with two different media and three *C. albicans* strains. Biofilms of these strains were grown in RPMI 1640 or Sabouraud dextrose broth (SDB) in the presence of the COX inhibitors. The results (Table 17) showed that none of these compounds inhibited biofilm formation (XTT activity) in RPMI 1640 or SDB, which indicates that the pK_a of COX inhibitors is not a factor in their activity against *Candida* biofilms.

Table 16. Effect of aspirin, salicylic acid and amphotericin B (AmB) on biofilm formation by *C. albicans* GDH 2346 in different media

Medium	Biofilm formation (XTT reduction %) *			
	Aspirin (1 mM)	Salicylic acid (1 mM)	AmB (2 μg/ml)	AmB (10 μg/ml)
YNB[d]	21.1 ± 4.2[a]	15.7 ± 3.0[a]	0.8± 0.3[a]	0.1 ± 0.1[a]
YNB+PBS[e]	62.1 ± 5.5[b]	77.9 ± 2.9[c]	0.3 ± 0.1[a]	0.2 ± 0.2[a]
YNB+MOPS[f]	101.0 ± 2.2	102.2 ± 1.2	0.1 ± 0.0[a]	0.2 ± 0.1[a]
RPMI+HEPES[g]	97.8 ± 3.9	95.0 ± 2.2	0.3 ± 0.2[a]	0.1± 0.1[a]

* Aspirin, salicylic acid and amphotericin B were present during the adhesion period and throughout the 48-h growth period. XTT reduction is expressed as a percentage of that of control biofilms incubated in the absence of any agent. Results are means ± SEM from at least two independent experiments carried out in triplicate. Mean (± SEM) control values (A_{492}) were: YNB, 2.548 ± 0.121; YNB+PBS, 2.873 ± 0.243; YNB+MOPS, 3.007 ± 0.101; RPMI+HEPS, 3.408 ± 0.073.

[a] Value significantly different at $P < 0.001$ from that of control.

[b] Value significantly different at $P < 0.01$ from that of control.

[c] Value significantly different at $P < 0.05$ for that of control.

[d] pH value of YNB started at 5.4 and ended at 3.4; with aspirin, pH value started at 4.6 and ended at 3.2.

[e] pH value of YNB+PBS started at 5.8 and ended at 5.3; with aspirin, pH value started at 5.7 and ended at 5.0.

[f] pH value of YNB+MOPS started at 7.1 and ended at 6.9.

[g] pH value of RPMI+HEPES started at 6.7 and ended at 6.4.

Table 17. Biofilm formation by three strains of *C. albicans* with COX inhibitors in two different media

Inhibitor (1 mM)	XTT reduction (%) [a] after 48 h					
	Strain GDH 2346		Strain SC5314		Strain CAI4	
	RPMI	SDB	RPMI	SDB	RPMI	SDB
Etodolac	94.0 ± 8.0	80.7 ± 2.0	104.3 ± 4.2	ND	111.1 ± 0.4	ND
Indomethacin	108.6 ± 2.3	102.6 ± 2.7	103.3 ± 7.2	ND	111.5 ± 0.1	ND
Piroxicam	107.9 ± 4.9	107.5 ± 8.0	108.5 ± 1.1	ND	108.1 ± 1.8	ND

[a] Inhibitors were present during the adhesion period and throughout the 48-h growth period. XTT reduction is expressed as a percentage of that of control biofilms incubated in the absence of any agent. Results are means ± SEM from at least two independent experiments carried out in triplicate. Mean (± SEM) control values (A_{492}) in RPMI+MOPS ranged from 2.969 ± 0.286 to 3.043 ± 0.384 for all strains, and for strain GDH 2346 in SDB was 2.391± 0.258.

ND, not determined.

5.5 Relationship between optical density and viability of planktonic cells of *C. albicans* GDH 2346 grown in buffered and unbuffered YNB in the presence of COX inhibitors

The NCCLS M27 macrodilution procedure is generally used to determine the MIC of an antifungal agent. However, here a similar protocol was used to determine the relationship between optical density and viability for planktonic cells grown in unbuffered YNB (Fig. 48), or YNB buffered with MOPS (Fig. 49), in the presence of COX inhibitors. All COX inhibitors significantly reduced the viability of planktonic cells grown in YNB (Fig. 48) but aspirin had the greatest effect, followed by salicylic acid and sodium salicylate (inhibition of 98 ± 0.6 %, 95.9 ± 0.5 % and 93 ± 0.4 %, respectively; $P<0.001$). Moreover, all COX inhibitors reduced the optical density of the culture by 23 ± 3.8 % to 47 ± 5.7 % ($P < 0.05$ to $P < 0.001$; Fig. 48).

In contrast, COX inhibitors did not significantly reduce the optical density or viability of planktonic cells grown in buffered YNB (Fig. 49), except for sodium salicylate and diclofenac which decreased cell viability by 38.2 ± 6.2 % and 11.9 ± 1.8 %, respectively ($P< 0.001$ and $P< 0.01$; Fig. 49). In these experiments, the pH value of the growth medium was measured in the presence of COX inhibitors before and after the 48-h incubation period, to determine whether the activity of COX inhibitors depended on pH. The results show that the initial pH of unbuffered YNB in the control (in the absence of COX inhibitors) was 5.3 and the final pH was 3.0 (Table 18), In the presence of COX inhibitors, the initial pH value was lower; for example, the pH values in the presence of salicylic acid and aspirin were 3.8 and 4.0, respectively. However, all the final pH values were

similar in the test cultures and the controls (grown with and without COX inhibitors) with range of pH values from 3.0 to 3.4 (Table 18). In contrast, there was no difference in the initial and final pH values when biofilms were grown in buffered YNB in the presence or absence of COX inhibitors. These results, taken together with the previous results (Figs. 48 and 49), confirm that the activities of COX inhibitors on *C. albicans* biofilms are pH dependent.

Figure 48

Relationship between optical density and viability of planktonic cells of *C. albicans* GDH 2346 grown in unbuffered YNB with different COX inhibitors present at 1 mM

The effect of different COX inhibitors (1 mM) on planktonic cells was evaluated by measuring viability (■) and optical density (▩).Viability is expressed as a percentage of that of control cells incubated under identical conditions in the absence of inhibitor. Viability results are means ± SEM of at least two experiments done in duplicate. Control counts gave mean values ranging from $2.5 \times 10^6 \pm 3.8 \times 10^3$ to $2.8 \times 10^6 \pm 3.8 \times 10^4$ cells /ml. Viable counts were carried out by the standard procedure of serial dilution followed by plating. Optical density of control cultures ranged from 1.401 to 1.427 at 520nm.

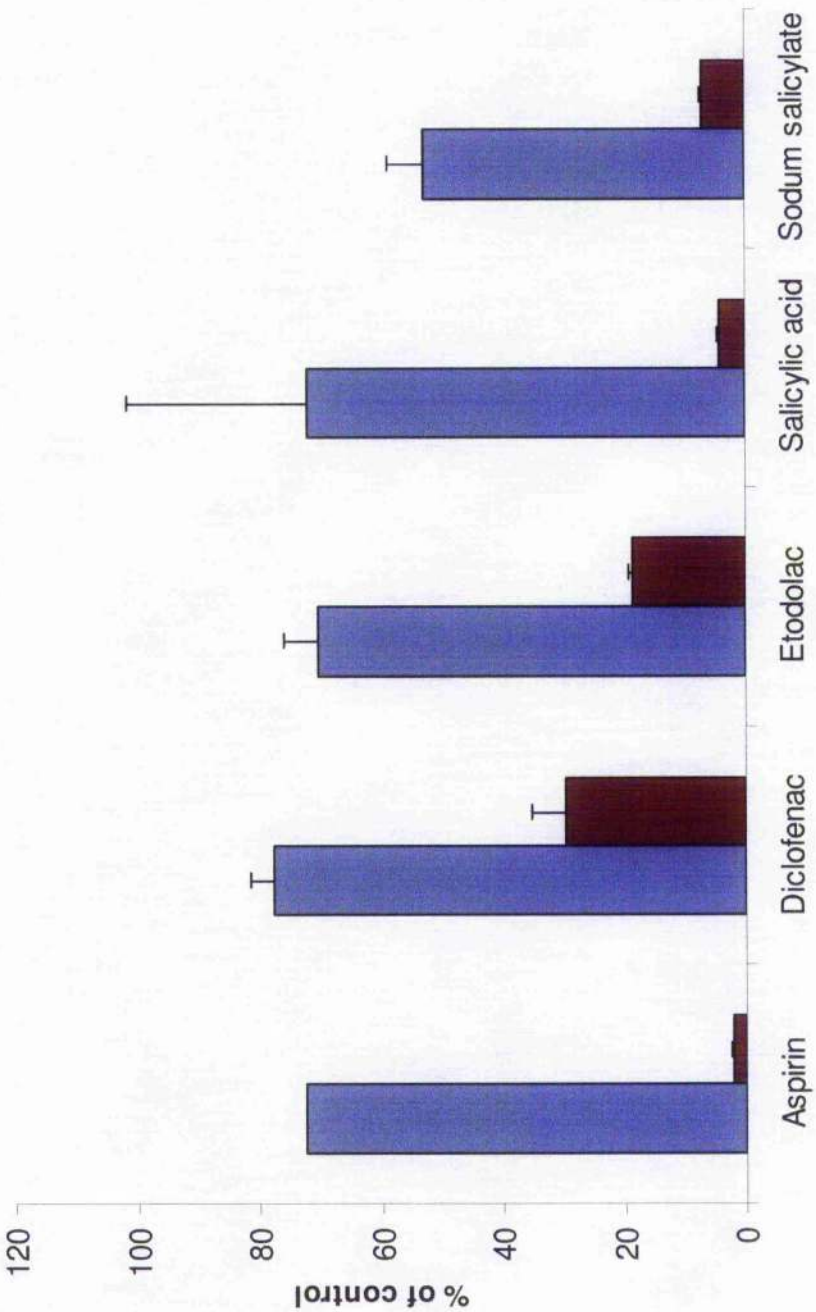

120
100
80
60
40
20
0
% of control
Aspirin
Diclofenac
Etodolac
Salicylic acid
Sodum salicylate

Figure 49

Relationship between optical density and viability of planktonic cells of *C. albicans* GDH 2346 grown in YNB+MOPS (pH 7) with different COX inhibitors present at 1 mM

The effect of different COX inhibitors (1 mM) on planktonic cells was evaluated by measuring viability (■) and optical density (■). Viability is expressed as a percentage of that of control cells incubated under identical conditions in the absence of inhibitor. Viability results are means ± SEM of at least two experiments done in duplicate. Control counts gave mean values ranging from $1.4 \times 10^6 \pm 5.5 \times 10^3$ to $1.6 \times 10^6 \pm 5.5 \times 10^3$ cells /ml. Viable counts were carried out by the standard procedure of serial dilution followed by plating. Optical density of control cultures ranged from 1.239 to 1.306 at 520nm.

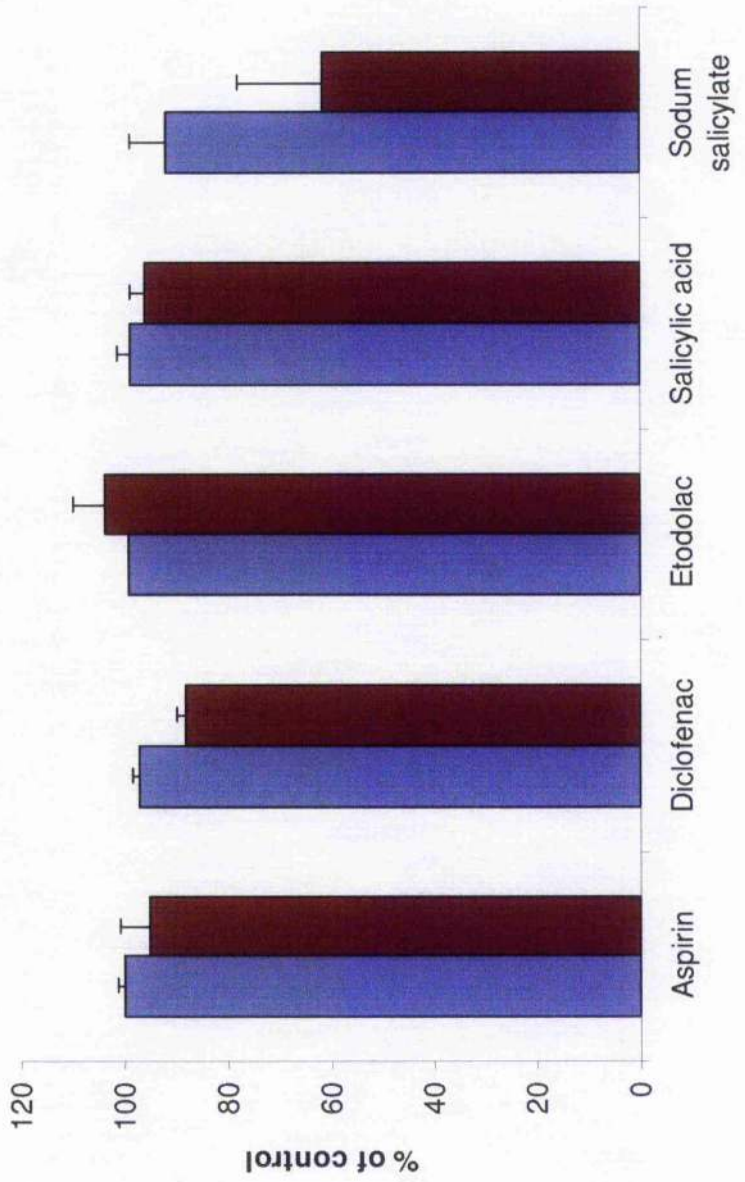

% of control
120
100
80
60
40
20
0
Aspirin
Diclofenac
Etodolac
Salicylic acid
Sodum salicylate

Table 18. pH measurements during planktonic growth of *C. albicans* GDH 2346 for 24h in YNB, with or without MOPS, and 1 mM COX inhibitor

Inhibitor (1 mM)	pH before and after growth in			
	YNB		YNB+MOPS	
	Initial pH	Final pH	Initial pH	Final pH
Aspirin	4.0	3.1	6.9	7.0
Diclofenac	5.5	3.2	6.9	7.0
Etodolac	4.4	3.0	6.9	7.0
Salicylic acid	3.8	3.0	6.9	7.0
Sodum salicylate	5.3	3.4	6.9	7.0

Results are means of two independent experiments. For controls grown in YNB the initial pH was 5.3 and the final pH was 3.0. For controls grown in YNB + MOPS both initial and final pH values were 7.0.

5.6 Effect of aspirin on the optical density and viability of planktonic cells of *C. albicans* GDH 2346 grown in different buffered media

As shown in the previous sections, COX inhibitors significantly affect planktonic cell viability during growth in unbuffered YNB. On other hand, aspirin had no effect when planktonic cells were grown in YNB buffered with MOPS at pH 7. In these experiments, further different types of buffer were used to determine whether the inhibitory activity of aspirin on optical density and viability could be influenced by the type of buffer used. Planktonic cells of *C. albicans* GDH 2346 were grown in YNB, YNB buffered with PBS or MOPS (pH 7) and RPMI buffered with MOPS (pH 7) in the presence or absence of 1 mM aspirin. After 24h the optical density and viability were determined. The results (Fig 50) confirm that the activity of COX inhibitors is not related to the type of buffer used in the medium, because 1 mM aspirin failed to affect cell viability in any buffered medium (Fig 50). However, it decreased cell viability in unbuffered YNB by 98 % as compared with controls grown in the absence of 1 mM aspirin ($P< 0.001$).

5.7 Relationship between the effect of 1 mM aspirin on optical density and the pH value of the growth medium for planktonic cells

It is clear from the previous results that most COX inhibitors had no significant effect on the viability and optical density of planktonic cells grown in buffered medium, irrespective of the type of buffer used. To determine whether aspirin activity was based on the pH value of the growth medium, its effect was investigated with planktonic cells grown in YNB buffered to different pH values. Planktonic cells of *C. albicans* GDH 2346 were grown for 48h in YNB medium buffered to pH values of 3, 4, 5, 6, 7 (0.1M citric acid- 0.2M disodium hydrogen phosphate buffer) and to pH 8 (0.2 M tris maleate-sodium hydroxide) buffer. The results showed that the final pH values of the growth medium with and without 1 mM aspirin were similar (Fig. 51A, B). At some pH values the optical density of aspirin-treated planktonic cells was lower than that of control cells grown in the absence of 1 mM aspirin (Fig. 51 A, B). When this decreased optical density was expressed as a percentage of the control optical density (Fig. 51 C) it was clear that aspirin had the greatest effects on cells grown in unbuffered YNB, or YNB buffered at pH 3, which showed an inhibition of 32.1 % and 24.2 %, respectively. The inhibitory effect of aspirin on optical density gradually decreased when planktonic cells were grown at pH values > 4. For example, at pH 5-7, aspirin had no effect on optical density (Fig 51, C).

5.8 Viable counts of planktonic and biofilm cells of *C. albicans* GDH 2346 grown at different pH values with 1 mM aspirin

To determine the effect of 1 mM aspirin on viability of planktonic cells and biofilms grown in YNB at different pH values, the growth medium was buffered

to pH 3, 4, 5, 6, 7 and 8. Aspirin was added at time zero for planktonic cells and during the adhesion period and again at time zero for biofilm formation. In both cases, incubation was for 48h. Figure 52 shows the correlation between aspirin activity and the pH value of the growth medium. Aspirin had the greatest effect (> 80%) on the viability of both planktonic and biofilm cells in unbuffered YNB, or YNB buffered to pH 3 ($P<0.001$) as compared with control cells grown at same pH value in the absence of aspirin. At pH 7, aspirin reduced biofilm cell viability by 35% ($P<0.01$; Fig. 52). These results confirm the previous findings with planktonic cells which showed that aspirin activity is pH dependent, and has the greatest effect on optical density at low pH values (Fig 51, C).

Figure 50

Effect of aspirin on the optical density and viability of planktonic cells of *C. albicans* GDH 2346 grown in different buffered media

The effect of 1 mM aspirin on planktonic cells grown in different buffered media was evaluated by measuring viability (■) and optical density (▩). Viability is expressed as a percentage of that of control cells incubated under identical conditions in the absence of aspirin. Viability results are means ± SEM of at least two replicate determinations. Control viable counts and O.D at 520nm gave mean values as follows: in YNB, $3.9 \times 10^6 \pm 1.5 \times 10^5$ with O.D 1.453; in YNB+PBS, $3.0 \times 10^6 \pm 1.0 \times 10^5$ with O.D 1.403; in YNB+MOPS, $3.2 \times 10^6 \pm 6.0 \times 10^4$ with O.D 1.413; in RPMI+MOPS, $4.2 \times 10^6 \pm 1.2 \times 10^5$ with O.D 1.581.

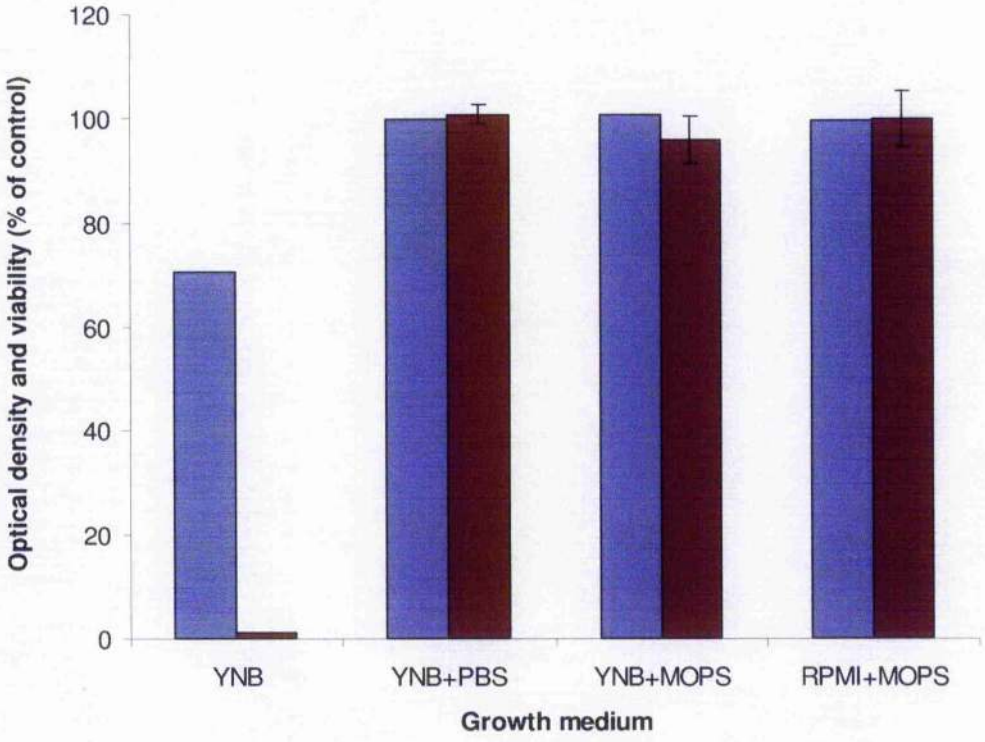
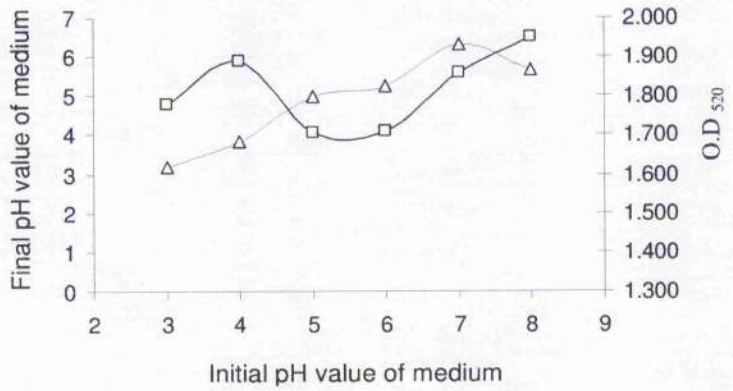

120
100
80
60
40
20
0
Optical density and viability (% of control)
YNB
YNB+PBS
YNB+MOPS
RPMI+MOPS
Growth medium

Figure 51

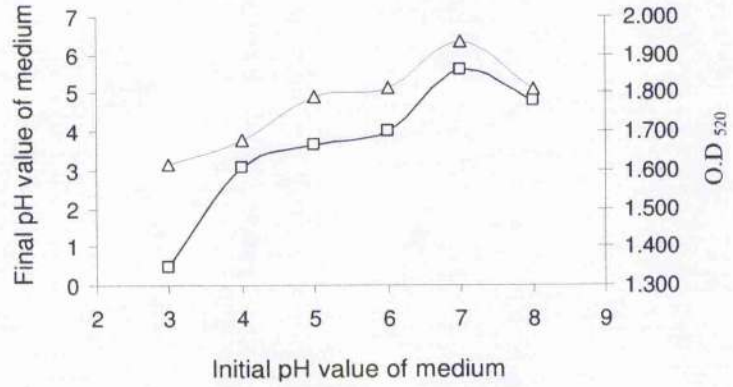
Effect of 1 mM aspirin on the optical density of *C. albicans* GDH 2346 cultures grown at different pH values

Planktonic cells of *C. albicans* GDH 2346 were grown for 48h in YNB medium buffered to different pH values ranging from 3 to 8 for 24h. The optical density (□) and final pH (△) were measured in the absence (A) or presence (B) of 1 mM aspirin. The inhibitory effect of aspirin on optical density was calculated as percentage of control growth in unbuffered YNB (C, open bar) or at identical pH values (C, closed bar). Results are from at least two experiments. The OD_{520} of the control culture in unbuffered YNB without 1 mM aspirin was 1.717.

A


Final pH value of medium
O.D 520
Initial pH value of medium

B


Final pH value of medium
O.D 520
Initial pH value of medium

C

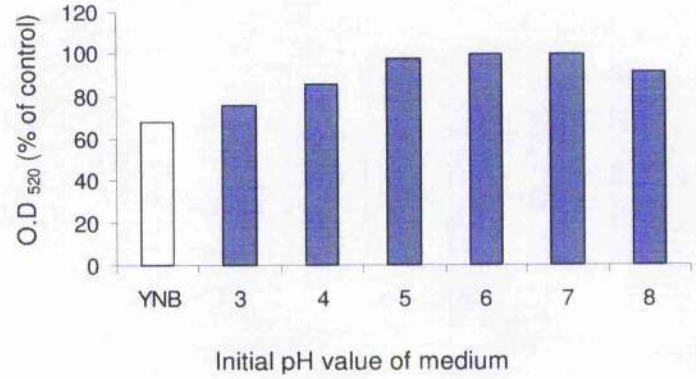
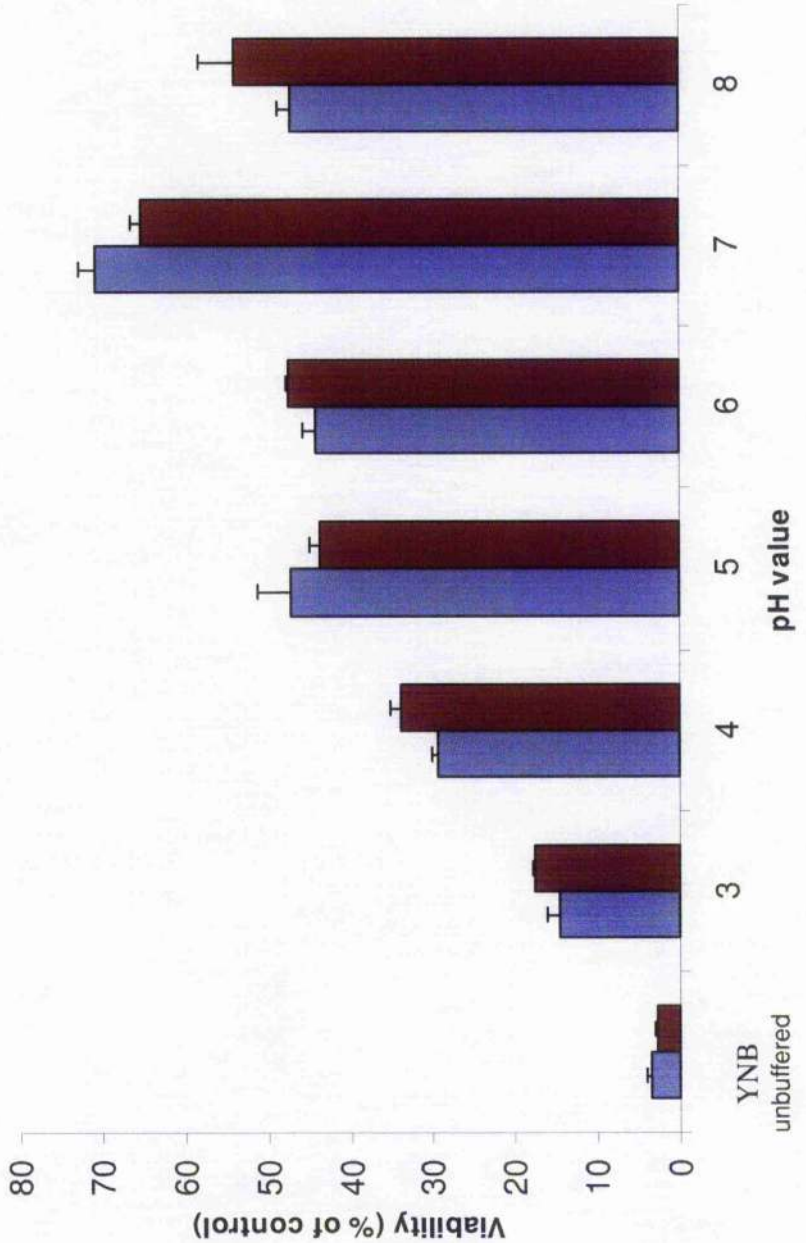

O.D 520 (% of control)
YNB
Initial pH value of medium

Figure 52

Viable counts of planktonic and biofilm cells of *C. albicans* grown at different pH values with 1 mM aspirin

Viability of planktonic (▩) or biofilm (■) cells is expressed as a percentage of that of control cells incubated at the same pH values in the absence of aspirin. Results are means ± SEM of at least two determinations. Control counts for planktonic cells ranged from $10.0 \times 10^6 \pm 1.2 \times 10^6$ to $17 \times 10^6 \pm 4.7 \times 10^4$ cells / ml and for biofilms from $0.9 \times 10^6 \pm 0.1 \times 10^6$ to $14 \times 10^6 \pm 0.3 \times 10^4$ cells / disk. Viable counts were carried out by the standard procedure of serial dilution followed by plating. For biofilm counts, cells from triplicate disks were resuspended in 3 ml of PBS then diluted and plated.


Viability (% of control)
0
10
20
30
40
50
60
70
80
YNB
unbuffered
3
4
5
6
7
8
pH value

5.9 Effects of COX inhibitors on metabolic activity of mature biofilms of *C. albicans* GDH 2346 grown in unbuffered YNB and YNB at pH 7

In YNB at pH 7, aspirin partially reduced biofilm viability (Section 5.8). In the light of this observation, a variety of different COX inhibitors were tested on mature biofilms grown in buffered YNB (pH 7). Mature (48-h) biofilms grown in the absence of COX inhibitors in YNB or YNB (pH 7) were transferred to identical, fresh medium containing different COX inhibitors at concentration of 1 mM and then incubated for a further period of 48h. Most of the COX inhibitors, and especially aspirin, significantly inhibited biofilm activity in unbuffered YNB (Table 19). In buffered YNB, aspirin inhibited biofilm metabolic activity by 27% ($P < 0.01$). The effects of diclofenac, ibuprofen and indomethacin were reasonably similar in buffered or unbuffered YNB, with inhibition of biofilm activity of 14 to 32% ($P < 0.01$ to $P < 0.05$; Table 19).

Table 19. Effect of COX inhibitors on XTT reduction by mature (48-h) biofilms of *C. albicans* GDH 2346

Inhibitor (1 mM)	XTT reduction (%)[a] after further incubation for 48h in	
	Unbuffered YNB	YNB buffered to pH 7
Aspirin	10.7 ± 2.1 [b]	73.4 ± 4.3 [c]
Diclofenac	67.7 ± 3.4 [c]	85.6 ± 4.8 [d]
Ibuprofen	78.5 ± 6.4 [d]	69.6 ± 2.3 [c]
Indomethacin	83.9 ± 3.4 [d]	77.8 ± 4.9 [d]
Piroxicam	90.6 ± 4.7	74.9 ± 4.9 [c]
Celecoxib	68.8 ± 3.7 [c]	87.9 ± 4.7
Etodolac	71.9 ± 2.0 [c]	93.3 ± 4.6
Nimesulide	97.2 ± 6.7	101.3 ± 4.0

[a] Different COX inhibitors were added to mature (48-h) biofilms grown in the absence of the drug in buffered or unbuffered YNB, and then the incubation was continued for a further 48 h. Biofilm activity (XTT reduction) after treatment with COX inhibitors is expressed as a percentage of that of control biofilms incubated in the same medium without inhibitors. Most results are means ± SEM of two independent experiments done in triplicate. The mean (± SEM) control value (A_{492}) was 2.616 ± 0.169 for biofilms grown in unbuffered YNB, and 2.630 ± 0.160 for biofilms grown in YNB buffered to pH 7 with 0.1M citric acid- 0.2M disodium hydrogen phosphate buffer.

[b] Value significantly different at $P < 0.001$ from that of control.

[c] Value significantly different at $P < 0.01$ from that of control.

[d] Value significantly different at $P < 0.05$ for that of control.

5.10 Effects of 1 mM aspirin on viability and metabolic activity of mature (48-h) *C. albicans* GDH 2346 biofilms grown in YNB buffered at different pH values

Metabolic activities of mature (48-h) biofilm were further evaluated at different pH values in the presence of 1 mM aspirin. The effect of aspirin on metabolic activity and viability of mature biofilms (Fig. 53) was pH dependent. For example, aspirin inhibited the metabolic activity of mature biofilms by more than 80% in unbuffered YNB or YNB buffered to pH 3 ($P < 0.001$), while at pH4 to pH 7 inhibition was 22 % to 43% ($P < 0.01$). Similar results were found for viability; aspirin reduced the viability of mature biofilms at pH 3 and pH 7 by 91 % and 14 % respectively (Fig. 53).

5.11 Effect of aspirin and fluconazole on metabolic activity of mature (48-h) *C. albicans* GDH 2346 biofilms grown at different pH values

Previous results confirmed that the activity of aspirin against developing biofilms (Fig 52) or mature biofilms (Fig. 53) of *C. albicans* was pH dependent. In these experiments, the activities of aspirin, fluconazole, and mixtures of the two drugs were compared against mature biofilms grown at different pH values. Aspirin at pH 3 inhibited the metabolic activity of mature biofilms by 88 %, more than either fluconazole or a mixture of fluconazole and aspirin. At pH 4, the combination of 1 mM aspirin with fluconazole (at 64 or 16 μg/ml) reduced the activity of mature biofilms by 40%, which was more than with fluconazole alone (P <0.05; Fig. 54). However, as expected, at pH 7 fluconazole inhibited the metabolic activity of mature *C. albicans* biofilms more than did aspirin.

Figure 53

Effects of 1 mM aspirin on viability and metabolic activity of mature (48-h) biofilms of *C. albicans* GDH 2346 grown at different pH values

Biofilms were grown for 48h without aspirin in YNB buffered at different pH values, transferred to fresh medium at the same pH value containing 1 mM aspirin (control biofilms were incubated without aspirin), and incubated for a further period of 48h. Results are means ± SEM of at least three replicates. Mean (± SEM) control values for XTT reduction (A_{492}) ranged from 2.213 ± 0.179 to 2.906 ± 0.043 for pH 3 to pH 7. Control viable counts gave mean values at pH 3 of $1.8 \times 10^6 \pm 0.7 \times 10^5$, and at pH 7 of $1.6 \times 10^6 \pm 0.6 \times 10^5$ cells / disk. For viable counts, cells from triplicate disks were resuspended in 3 ml of PBS then diluted and plated.

■ XTT reduction

■ Viability (done for pH 3 and pH 7 only)

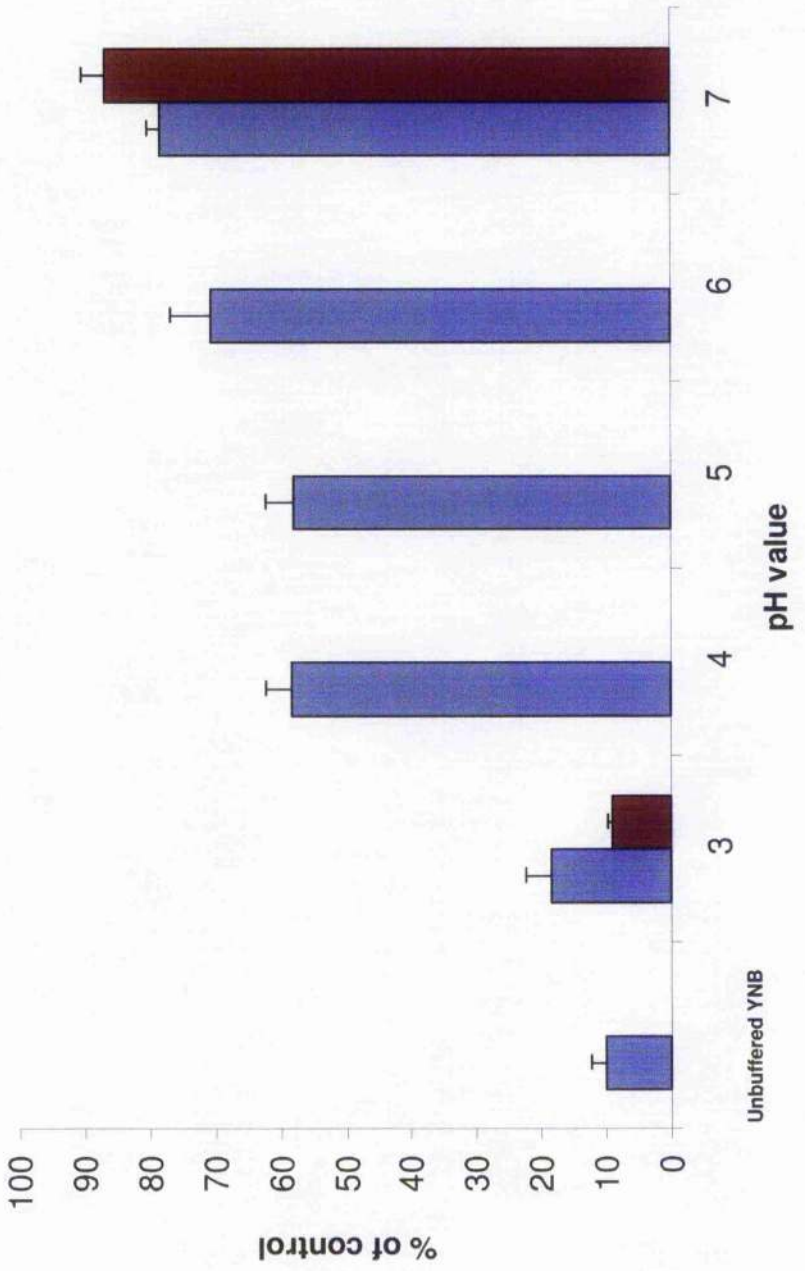
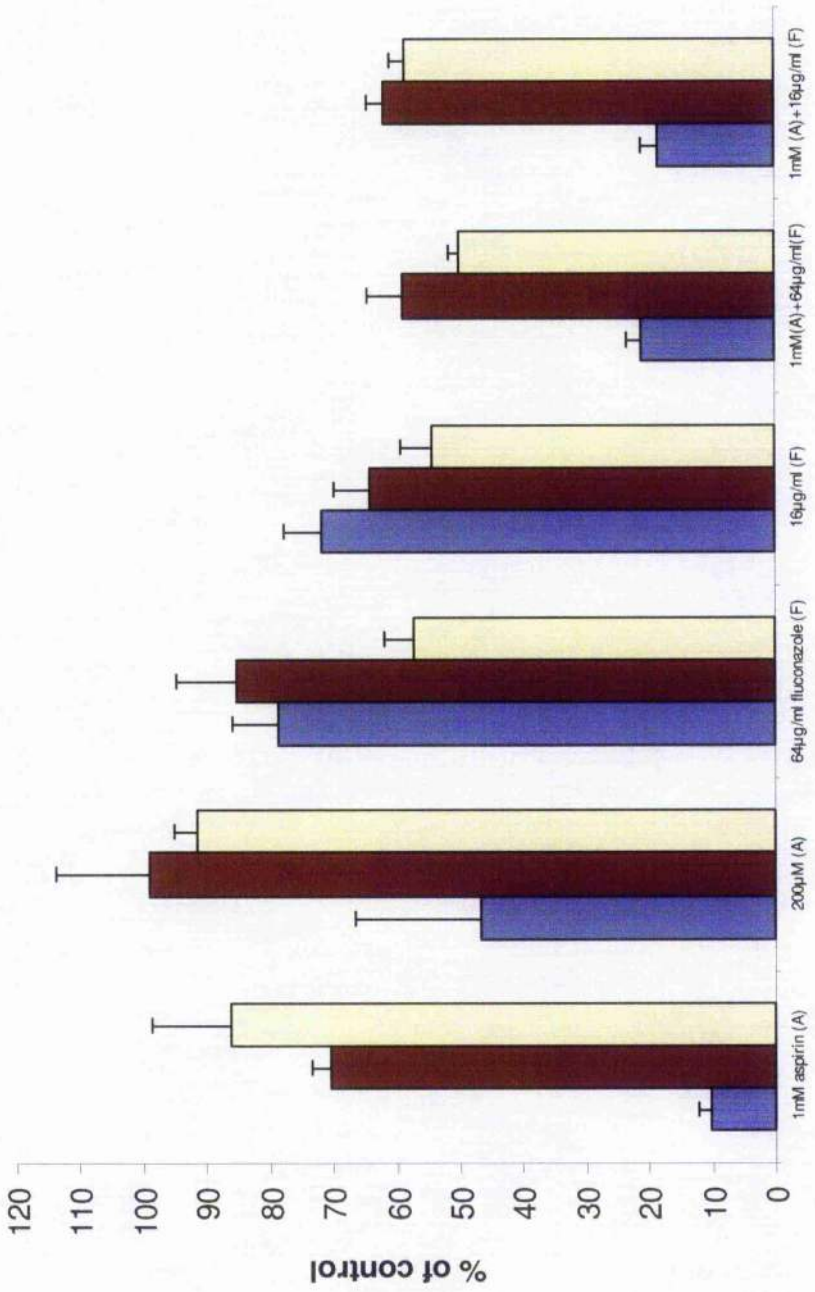

% of control
100
90
80
70
60
50
40
30
20
10
0
Unbuffered YNB
3
4
5
6
7
pH value

Figure 54

Effects of aspirin and fluconazole on metabolic activity of mature (48-h) biofilms of *C. albicans* GDH 2346 grown at different pH values

Mature 48-h biofilms were grown in the absence of any agent in YNB buffered to pH 3 (■), pH 4 (■) or pH 7 (□). Biofilms were then transferred to fresh medium at the same pH and incubated for a further period of 48h in the presence or absence of aspirin and/ or fluconazole. Results are means ± SEM from at least three replicates. The mean (± SEM) control values for XTT reduction (A_{492}) were 2.479 ± 0.224, 2.593 ±0.048 and 3.025 ± 0.126 for pH 3, 4 and 7, respectively.


% of control
120
110
100
90
80
70
60
50
40
30
20
10
0
1mM aspirin (A)
200µM (A)
64µg/ml fluconazole (F)
16µg/ml (F)
1mM(A)+64µg/ml(F)
1mM (A)+16µg/ml (F)

5.12 Effects of aspirin and fluconazole on metabolic activity of mature (48-h) biofilms of *C. albicans* GDH 2346 grown in three different media

Since the pH value of the growth medium clearly plays an important role in the antifungal activity of aspirin, it was of interest to determine the effects of aspirin and fluconazole on mature biofilms grown in synthetic vagina-simulative (VS) medium pH 4.2 which was developed as vaginal fluid simulant to investigate therapeutic products (Owen and Katz, 1999). This medium was used to evaluate fluconazole fungicidal activity in a previous study (Moosa *et al.*, 2004), which demonstrated that fluconazole was fungicidal for *C. albicans* in VS, but not in other media at pH 4.2.

In this study, the effects of aspirin and fluconazole were investigated on mature biofilms of *C. albicans* GDH 2346 grown in unbuffered YNB, YNB + 17 mM acetic acid at pH 4.2, and vagina-simulative (VS) medium, pH 4.2. The results show that 1 mM aspirin inhibited mature biofilms grown in YNB medium by up to 90 % which is significantly more than the effect of aspirin in either YNB + 17 mM acetic acid or in synthetic vagina-simulative (VS) medium ($P<0.001$; Fig. 55). Moreover, mature biofilms grown in YNB + 17 mM acetic acid were inhibited by 1 mM aspirin by 63 % ($P<0.01$) which is more than the inhibition by 64 or 16 μg fluconazole at 35 % and 45%, respectively (Fig 55).

5.13 Effects of aspirin and fluconazole on metabolic activity of mature (48-h) biofilms of *C. glabrata* AAHB 12 grown in three media

It is known that *C. glabrata* is normally resistant to fluconazole (Pfaller *et al.*, 2001). In the view of this fact, mature biofilms of *C. glabrata* AAHB 12 were

tested in the presence of aspirin and fluconazole individually, or in combination. The results show that 1 mM aspirin alone inhibited mature biofilms of *C. glabrata* grown in all three media by up to 75 % (P <0.001; Fig. 56). This effect was greater than that of 64 μg fluconazole which inhibited metabolic activity of mature biofilms grown in VS medium by 50 % (P<0.01; Fig. 56).

5.14 Viability of mature (48-h) biofilms of *C. albicans* GDH 2346 and *C. glabrata* AAHB 12 after treatment with 200 μM or 1 mM aspirin

As described in Section 5.12, the effect of aspirin on *C. albicans* GDH 2346 was greater in YNB medium (> 90 % inhibition) than in either YNB + 17 mM acetic acid or in synthetic vagina-simulative (VS) medium. However, with mature biofilms of *C. glabrata*, aspirin inhibited metabolic activity by about 75-80 % in all three media (Fig. 56; Section 5.13). In these experiments the viabilities of biofilms of both species were investigated after aspirin treatment. Viable counts were carried out after further incubation of mature biofilms (48-h) of *C. albicans* GDH 2346 and *C. glabrata* AAHB 12 in the presence of 200μM or 1 mM aspirin. As expected, the effect of 1 mM aspirin on *C. albicans* biofilms was greater in unbuffered YNB (90 % loss of viability; P <0.001) than in YNB + 17 mM acetic acid and VS medium (58 % and 45% loss of viability, respectively; Table 20). For *C. glabrata* AAHB 12, 1 mM aspirin decreased the viability of mature biofilms by more than 60% in YNB + 17 mM acetic acid or in VS medium. Furthermore, in unbuffered YNB there was a 90 % loss of viability following treatment with 1 mM aspirin (P < 0.001; Table 20).

Figure 55

Effects of aspirin and fluconazole on metabolic activity of mature (48-h) biofilms of *C. albicans* GDH 2346 grown in three different media

Mature 48-h biofilms were grown in the absence of any agents in YNB (■) , YNB+ acetic acid (■) and vagina-simulative (VS) medium (□). Biofilms were then transferred to identical, fresh medium and incubated for a further period of 48h in the presence or absence of aspirin and/or fluconazole. Results are means ± SEM of two experiments with at least three replicates each time. The mean (± SEM) control values for XTT reduction (A_{492}) were 2.552 ± 0.069, 2.546 ± 0.118 and 1.567 ± 0.077 for YNB, YNB+ acetic acid, and vagina-simulative (VS) medium, respectively.

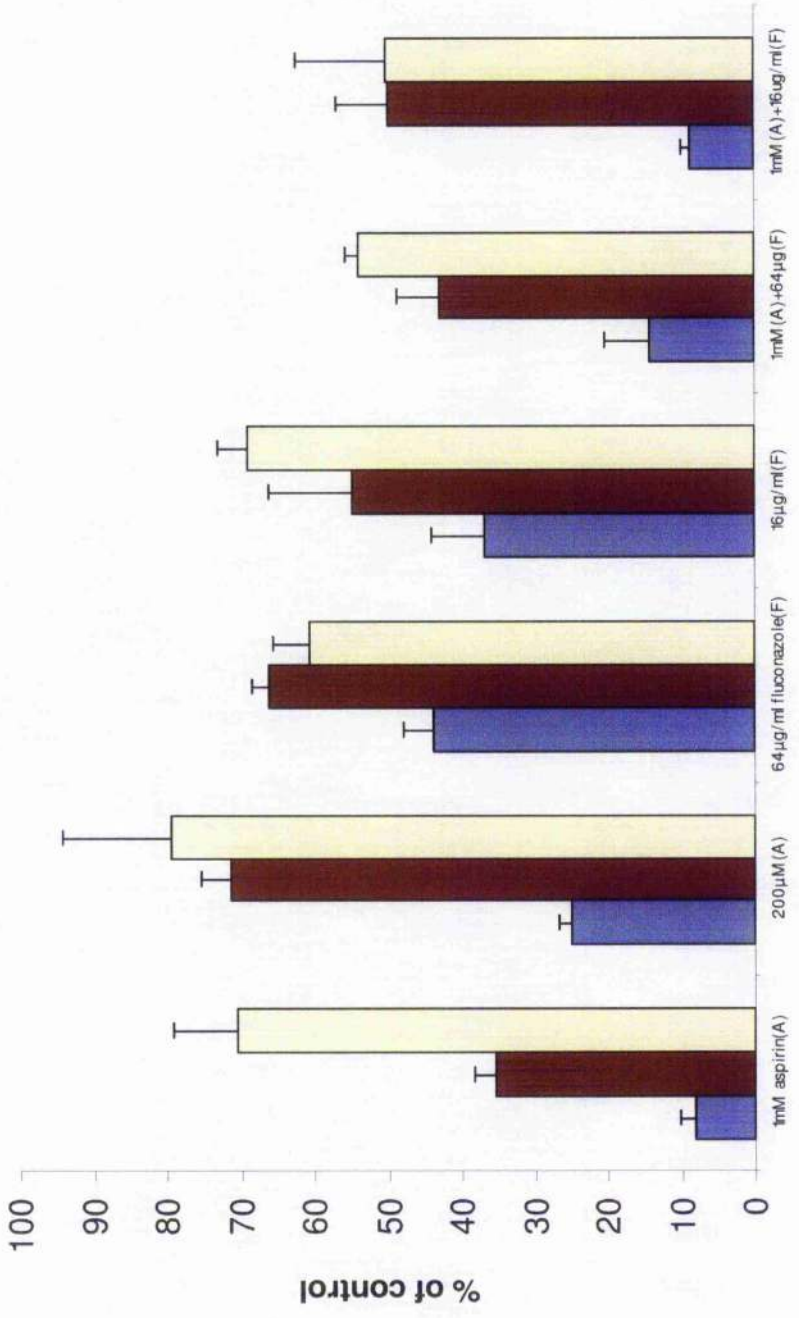

% of control
100
90
80
70
60
50
40
30
20
10
0
1mM aspirin(A)
200µM(A)
64µg/ml fluconazole(F)
16µg/ml(F)
1mM(A)+64µg(F)
1mM(A)+16ug/ml(F)

Figure 56

Effects of aspirin and fluconazole on metabolic activity of mature (48-h) biofilms of *C. glabrata* AAHB 12 grown in three media

Mature 48-h biofilms were grown in the absence of any agent in YNB (■) , YNB+ acetic acid (■) and vagina-simulative (VS) medium (□). Biofilms were transferred to identical, fresh medium and then incubated for further period of 48h in the presence or absence aspirin and/or fluconazole. Results are means ± SEM from two experiments with as least three replicates each time. The mean (± SEM) control values for XTT reduction (A_{492}) were 1.661 ± 0.108, 1.569 ± 0.116 and 0.739 ± 0.073 for YNB , YNB+ acetic acid and vagina-simulative (VS) medium, respectively.

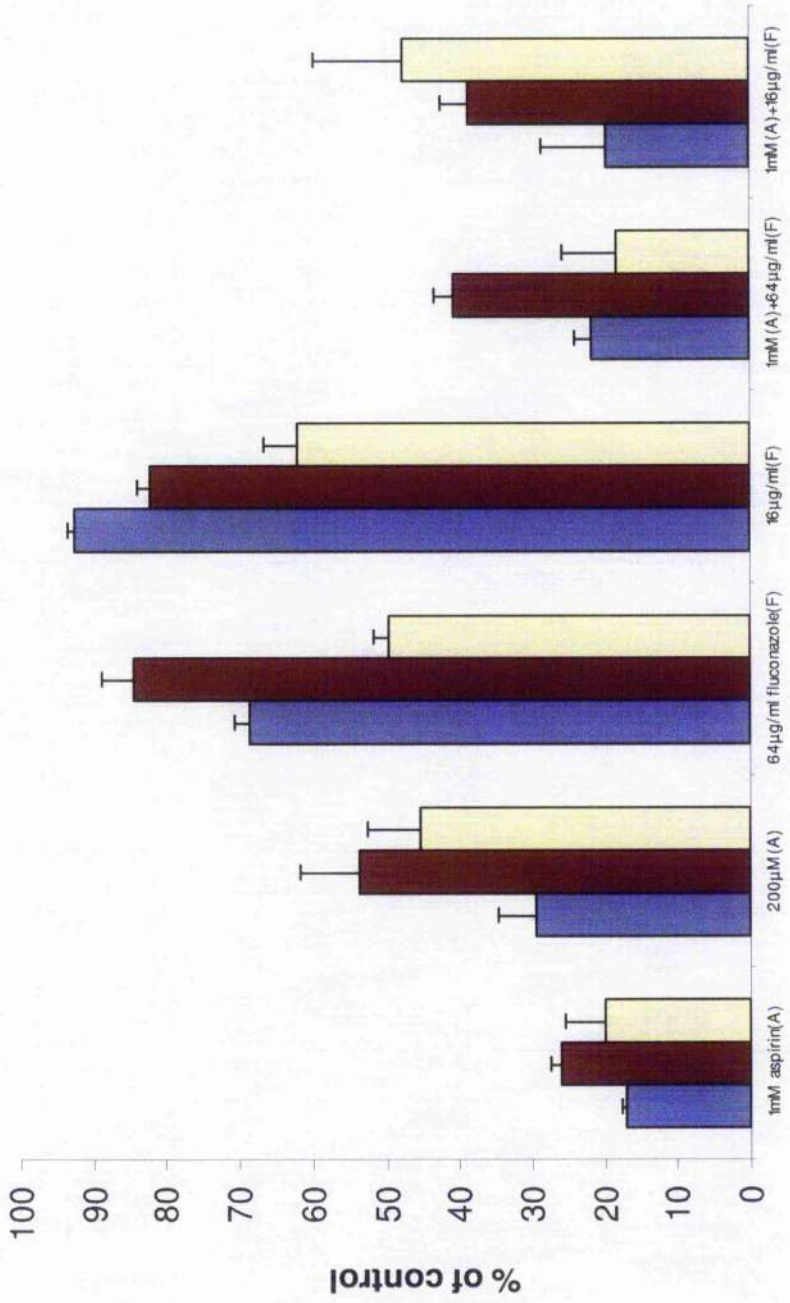

% of control
100
90
80
70
60
50
40
30
20
10
0
1mM aspirin(A)
200µM (A)
64µg/ml fluconazole(F)
16µg/ml(F)
1mM (A)+64µg/ml(F)
1mM (A)+16µg/ml(F)

Table 20. Viability of mature (48-h) biofilms of *C. albicans* GDH 2346 and *C. glabrata* AAHB 12 after treatment with 200 μM or 1 mM aspirin

Medium	Viability (%)			
	C. albicans GDH 2346		*C. glabrata* AAHB 12	
	Aspirin (200 μM)	Aspirin (1 mM)	Aspirin (200 μM)	Aspirin (1 mM)
YNB	20.0 ± 1.1 [c]	8.6 ± 1.5 [a]	10.2 ± 0.4 [a]	6.7 + 0.5 [a]
YNB [HAc]	51.8 ± 8.6 [d]	41.9 ± 2.7 [d]	67.3 ± 6.1 [d]	27.3 ± 2.1 [c]
VS medium	68.9 ± 2.3 [d]	54.7 ± 2.7 [d]	55.8 ± 1.1 [d]	34.3 ± 1.9 [c]
YNB pH 7	ND	ND	70.0 ± 1.7 [d]	64.0 ± 2.0 [d]

Mature, 48-h biofilms were grown in the absence of aspirin in different media and then transferred to fresh identical medium containing 200 μM or 1 mM aspirin and incubated for a further period of 48h. Viability is expressed as a percentage of that of control cells incubated under identical conditions in the absence of aspirin and fluconazole. Results are means ± SEM of at least two determinations. Control counts for *C. albicans* GDH 2346 gave mean values of $0.32 \times 10^6 \pm 3.2 \times 10^3$ to $1.22 \times 10^6 \pm 5 \times 10^3$ cells / biofilm disk, and for *C. glabrata* $1.9 \times 10^6 \pm 4.1 \times 10^4$ to $4.3 \times 10^6 \pm 8.5 \times 10^4$. For viable counts, cells from triplicate disks were resuspended in 3 ml of PBS, then diluted and plated.

[a] Value significantly different at $P < 0.001$ from that of control.

[b] Value significantly different at $P < 0.01$ from that of control.

[c] Value significantly different at $P < 0.05$ for that of control.

YNB [HAc], YNB medium containing 17 mM acetic acid.

ND, not determined.

6. Prostaglandin production during growth of *C. albicans* biofilms

Prostaglandin synthesis and its sensitivity to COX inhibitors during growth of planktonic cells and biofilms were investigated using a prostaglandin-screening enzyme immunoassay kit (Cayman Chemicals). This ELISA detects PGE_2, PGE_1, $PGF_{2\alpha}$, and $PGF_{1\alpha}$, and to a lesser extent $PGF_{3\alpha}$, PGD_2, PGE_3, and thromboxane B_2. It does not detect the PGA class, PGB_1, 15-keto PGE_2, 13,14-dihydro-15-keto $PGF_{2\alpha}$, or misoprostol.

6.1 Development of prostaglandin standard curve using growth medium

A prostaglandin standard curve was prepared using YNB medium (Fig. 57 A). The results showed that there was no interference between YNB medium and the ELISA assay. Moreover, as can be seen from Fig. 57 A, the ELISA kit can detect prostaglandins in the range of 15 to 2000 pg/ml. Preliminary experiments showed that low levels of prostaglandin were detected in the supernatant of planktonic cells of *C. albicans* GDH 2346 grown in YNB for 48h. YNB medium was therefore replaced by Sabouraud dextrose broth (SDB) in an attempt to increase prostaglandin secretion. The results showed that one or more components of SDB medium interfered with the assay (Fig 57 B). Further experiments revealed that neopeptone (a component of SDB) from two different sources interfered with the assay (data not shown). In addition, 1 mM arachidonic acid, which has been used previously to promote prostaglandin secretion by *C. albicans* (Noverr *et al.*, 2001), appeared to interfere with this assay. Routinely, therefore, culture supernatants from either biofilms or planktonic cells were concentrated 10-fold by freeze-drying before the prostaglandin assay since preliminary

experiments showed that a ten-fold concentrate is the maximum that can be used with this kit without interference (Fig. 57C).

6.2 Prostaglandin production by biofilms and planktonic cells of *C. albicans* GDH 2346

Prostaglandin synthesis was barely detectable after incubation for 10 h, at concentrations of 1.4 ± 0.8 pg/ml (mean ± SEM) and 0.5 ± 0.5 pg/ml for planktonic and biofilm cells, respectively (Fig. 58 A, B). After 10 h, however, prostaglandin production increased rapidly to a maximum of 61.9 ± 2.7 pg/ml for planktonic cells and 49.1 ± 2.7 pg/ml for biofilm cells after 48 h. Throughout the entire growth period there was little correlation between prostaglandin concentration and cell density. When prostaglandin production was calculated as a function of cell dry weight, it became apparent that biofilm cells produce more prostaglandin than do planktonic cells. For example, at 24 and 48h biofilms produced 38.5 ± 11.3 and 95.5 ± 19.7 (pg/mg dry wt), respectively. This is significantly more ($P<0.05$) than the prostaglandin concentrations of 14.6 ± 0.4 and 51.8 ± 2.3 (pg/mg dry wt) detected with planktonic cells at the same time periods (Fig. 58 C).

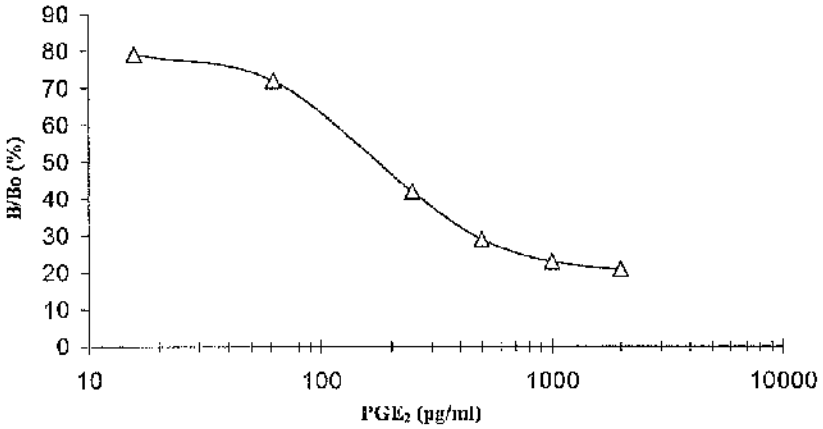
Figure 57

Prostaglandin standard curve for the prostaglandin screening kit

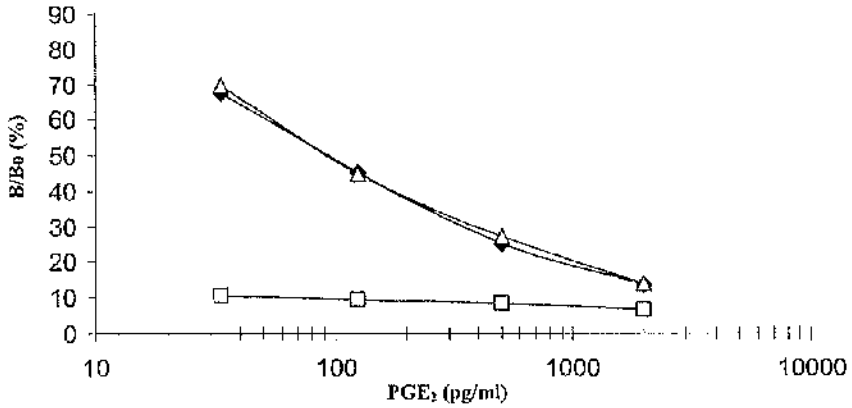
Prostaglandin (PGE_2) solutions were prepared according to the manufacturers instructions in (A) YNB; (B) EIA reagent ◆ , YNBΔ or SDB □ ; and (C) a 10-fold concentrate of YNB.

EIA , Buffer supplied with ELISA kit.
B, Sample or standard absorbance reading at A_{405} .
Bo, Maximum absorbance reading at A_{405} from positive control.

A. Standard curve of PGE_2 in YNB



B. Standard curve of PGE_2 in EIA, YNB and SDB



C. Standard curve of PGE_2 in ten-fold concentrate of YNB

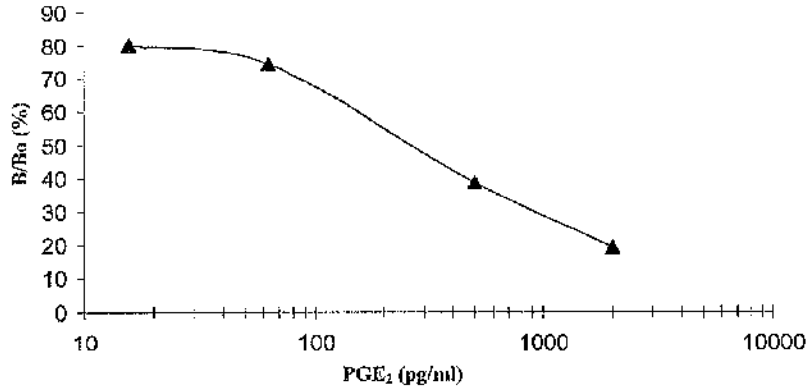
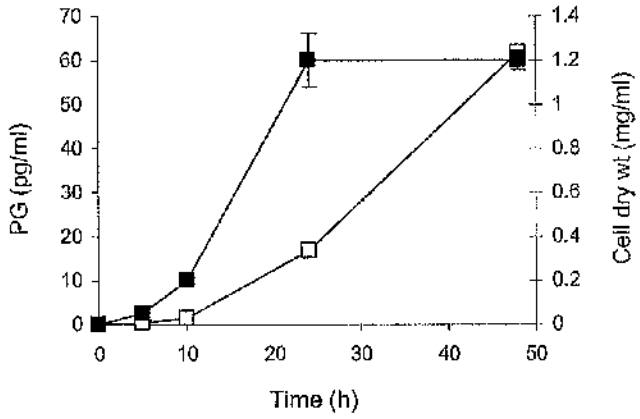


Figure 58

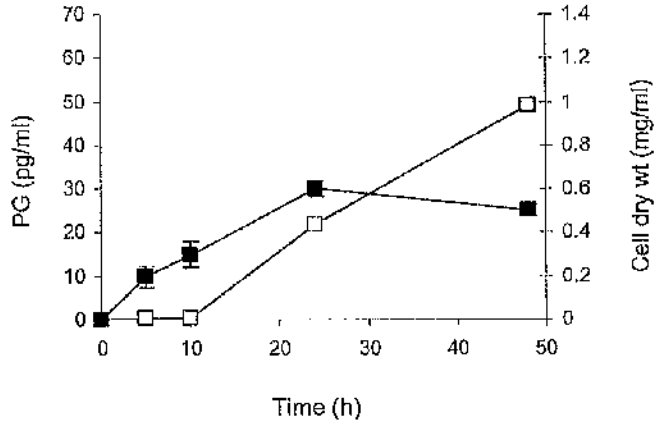
Prostaglandin production by biofilms and planktonic cells of *C. albicans* GDH 2346

Relationship between prostaglandin production (□) and cell dry weight (■) of (A) planktonic cells and (B) biofilms of *C. albicans* GDH 2346 during growth over 48 h. The results are means ± SEM of three independent experiments carried out in duplicate. (C) Prostaglandin production expressed as a function of cell dry weight for planktonic cells (○) and biofilms (●) of *C. albicans* GDH 2346. The results are means ± SEM of three independent experiments carried out in duplicate.

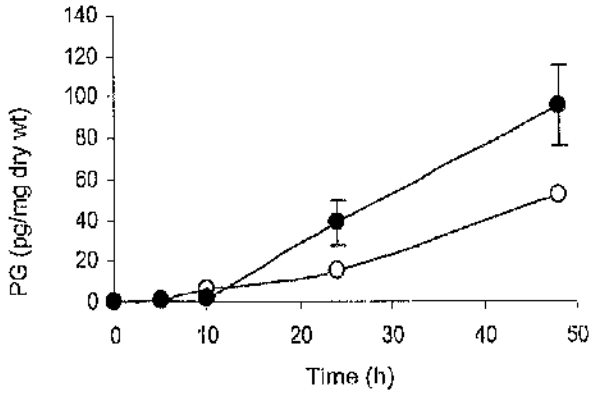
A


PG (pg/ml)
Cell dry wt (mg/ml)
Time (h)
0
10
20
30
40
50
60
70
0.2
0.4
0.6
0.8
1
1.2
1.4

B


PG (pg/ml)
Cell dry wt (mg/ml)
Time (h)
0
10
20
30
40
50
60
70
0.2
0.4
0.6
0.8
1
1.2
1.4

C


PG (pg/mg dry wt)
Time (h)
0
20
40
60
80
100
120
140
10
30
50

6.3 Effects of COX inhibitors on prostaglandin production by biofilms and planktonic cells of *C. albicans* GDH 2346

Previous work showed that aspirin, diclofenac and etodolac had the greatest inhibitory effects on the growth of *Candida* biofilms (section 4.2). In this part of the project, these inhibitors were used at a relatively low concentration of 50 μM. Aspirin at this concentration was shown by the earlier work to decrease biofilm formation by about 20% after 48 h (section 4.3). Here, aspirin significantly reduced prostaglandin synthesis by both biofilms and planktonic cells (Table 21). For biofilms, prostaglandin production was only 48.6% of that of control cells after 24 h ($P < 0.001$), but had recovered to 77.4% after 48 h ($P < 0.01$). Both diclofenac and etodolac also significantly decreased prostaglandin synthesis by biofilms after 48 h ($P < 0.05$).

6.4 Prostaglandin production by *C. albicans* SC5314 and its morphological mutant HLC54 (*cph1/cph1 efg1/efg1*)

To investigate further a possible role for *Candida* prostaglandin in hyphal formation during biofilm development, prostaglandin production was determined in a mutant with defined defects in two filamentation pathways (*cph1/cph1 efg1/efg1*) and its wild-type strain *C. albicans* SC5314. The mutant is capable of growth in the yeast form only, whereas the parent strain can grow in both yeast and hyphal forms. Prostaglandin levels were measured for biofilm and planktonic cells of both strains after 24 and 48 h. As noted already with strain GDH 2346, biofilm cells of both wild type and mutant produced significantly more prostaglandin than did planktonic cells ($P < 0.001$ to $P < 0.05$; Fig. 59). For example, biofilm cells of strain SC5314 secreted 234.2 ± 0.6 pg/mg dry wt (mean

± SEM) after 48 h whereas planktonic cells of the same strain produced only 53.8 ± 3.9 pg/mg. However, there was no significant difference between prostaglandin production by biofilm cells of the mutant and parent strains after 48 h (Fig. 59).

6.5 Scanning electron microscopy of *C. albicans* SC5314 and its morphological mutant HLC54 (*cph1/cph1 efg1/efg1*)

Scanning electron microscopy demonstrated that biofilms of the parent strain possessed a morphology typical of *C. albicans* biofilms on PVC catheter disks ie., a basal region of densely packed yeast cells with an overlying, mostly hyphal layer (Fig 60A; Baillie & Douglas, 1999b). The mutant also produced substantial biofilms, but in this case the structures consisted of yeast cells only (Fig. 60B). Overall, our experiments with the morphological mutant demonstrate that the genetic defects of this strain did not affect its ability to secrete prostaglandin. Moreover, biofilms of this strain, consisting entirely of yeast cells, produced significantly more prostaglandin than did planktonic cells.

Table 21. Effects of COX inhibitors on prostaglandin production by biofilms and planktonic cells of *C. albicans* GDH 2346

Growth condition	Prostaglandin production (%)	
	Planktonic cells	Biofilm
24h with Aspirin	76.2 ± 6.2[a]	48.6 ± 3.7[a]
24h with Diclofenac	86.9 ± 3.4[c]	100.0 ± 8.5
24h with Etodolac	84.1 ± 6.2[a]	93.7 ± 11.1
48h with Aspirin	75.3 ± 8.9[a]	77.4 ± 26.4[b]
48h with Diclofenac	81.5 ± 9.2	88.4 ± 17.7[c]
48h with Etodolac	89.9 ± 1.4[a]	82.2 ± 10.8[c]

Inhibitors were present at a concentration of 50 μM throughout the incubation period (24h or 48h). Prostaglandin production in the presence of inhibitor, determined as pg/mg cell dry weight, is expressed as the percentage of that produced by control cells incubated in the absence of inhibitor. Results are means ± SEM of at least two independent experiments carried out in duplicate. Mean ± SEM values for controls were 14.6 ± 0.4 pg/mg and 51.8 ± 2.76 pg/mg for planktonic cells at 24h and 48h, respectively; and 38.5 ± 11.1 pg/mg and 95.5 ± 19.7 pg/mg for biofilm cells at 24h and 48h, respectively.

[a] Value significantly different at $P < 0.001$ from that for the control.

[b] Value significantly different at $P < 0.01$ from that for the control.

[c] Value significantly different at $P < 0.05$ from that for the control.

Figure 59

Prostaglandin production by *C. albicans* SC5314 and its morphological mutant HLC54 (*cph1/cph1 efg1/efg1*)

Prostaglandin production after 24 h (white bars) and 48 h (black bars) by planktonic cells and biofilms of *C. albicans* SC5314 and its morphological mutant HLC54 (*cph1/cph1 efg1efg1*).The results are means ± SEM of two independent experiments carried out in duplicate.

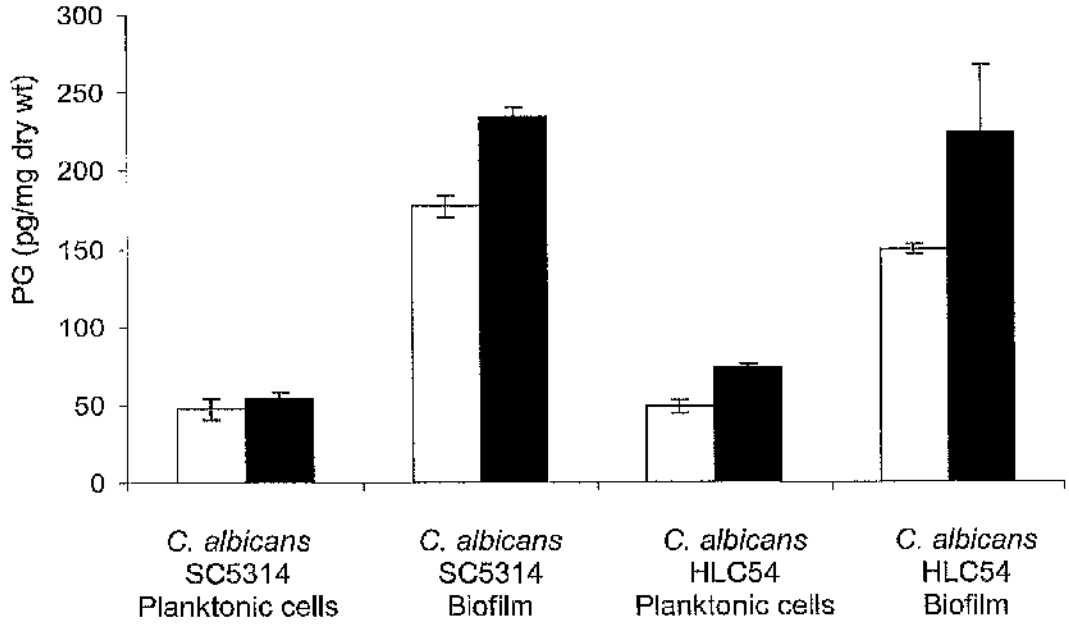
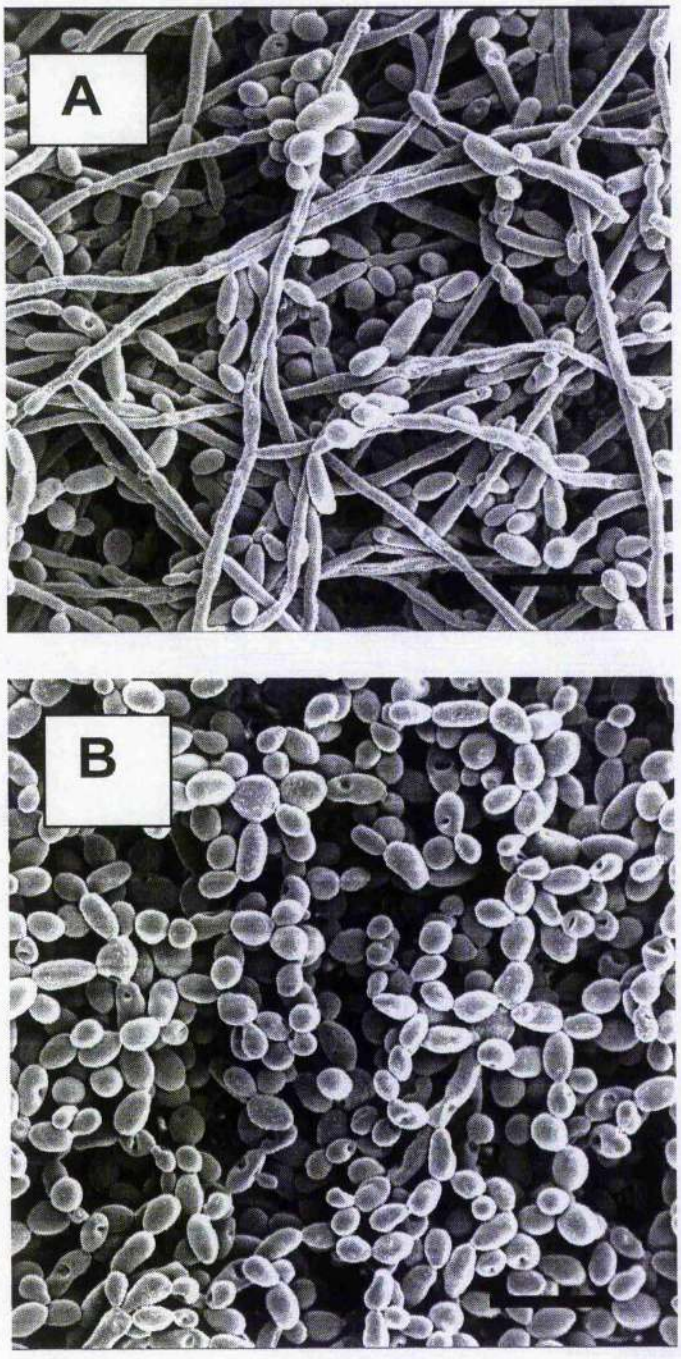

PG (pg/mg dry wt)
300
250
200
150
100
50
0
C. albicans SC5314 Planktonic cells
C. albicans SC5314 Biofilm
C. albicans HLC54 Planktonic cells
C. albicans HLC54 Biofilm

Figure 60

Scanning electron microscopy of *C. albicans* SC5314 and its morphological mutant HLC54 (*cph1/cph1 efg1/efg1*)

SEM of 48-h biofilms of *C. albicans* SC5314 (A) and its morphological mutant HLC54 (*cph1/cph1 efg1/efg1*) (B). Bars, 10 μm.


A
B

7. Effects of steroids on biofilm development and germ-tube formation by *C. albicans*

Hormones or pheromones have been described in unicellular organisms. For example, fungal sex hormones are responsible for bringing fungal cells together in a physiological state for mating (Gooday and Adams, 1993); additionally, fungi appear to interact with mammalian hormones at a level closely analogous to the interaction of mammalian cells with these chemical molecules. Therefore in this study the effects of steroids on *C. albicans* biofilm development and germ-tube formation was investigated.

7.1 Biofilm formation in the presence of steroids

The steroids used were progesterone, corticosterone, hydrocortisone, dexamethasone and prednisolone. The effects of different concentrations of all these compounds were evaluated against biofilms grown on catheter disks. Steroids were added individually at time zero of biofilm formation by *C. albicans* GDH 2346. The effect of a wide range of steroid concentrations (0.01 to 1000 μM) was explored (Fig. 61). The metabolic activity (XTT reduction) of *C. albicans* biofilms was not affected by any of these compounds. For example, 1 mM progesterone or hydrocortisone slightly inhibited biofilm formation (by 14%), but this inhibition was not significant ($P>0.05$). Similar results were found for all steroids tested at all concentrations.

7.2 Effect of steroids on germ-tube formation by *C. albicans*

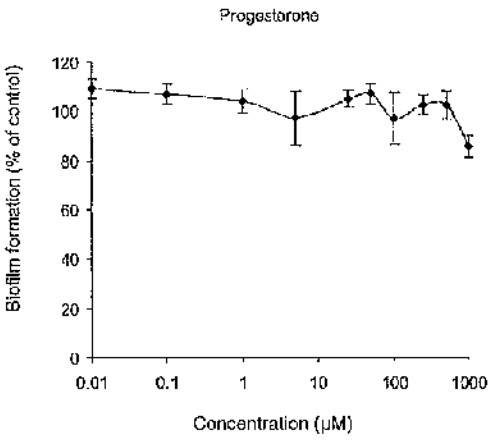
As shown previously, steroids did not significantly affect the metabolic activity of *C. albicans* biofilms. In these experiments the effect of steroids on

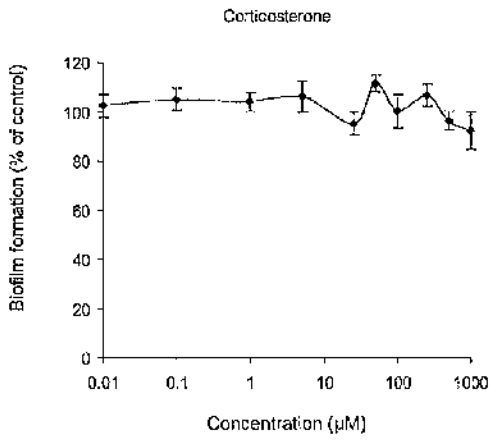
germ-tube formation was investigated. Germ tubes were allowed to form for 5h in 10 mM proline + 2.5 mM N-acetylglucosamine buffered with 50 mM potassium phosphate (pH 6.5) in the presence or absence of different steroids. Five different concentrations of each steroid were tested. None of the steroids significantly inhibited germ-tube formation at 0.01 or 0.1 μM. However, at 1 μM, progesterone and dexamethasone inhibited germ-tube formation by 34 and 25 %, respectively ($P<0.05$; Table 22). At 100 to 1000 μM, all steroids inhibited germ-tube formation. In particular, corticosterone and prednisolone at 1000 μM inhibited germ-tube formation by 64-65 % ($P<0.01$; Table 22).

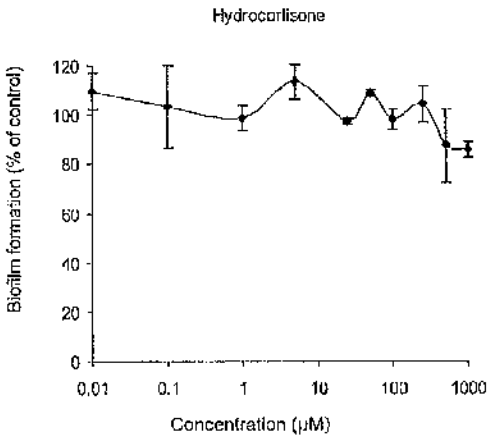
Figure 61

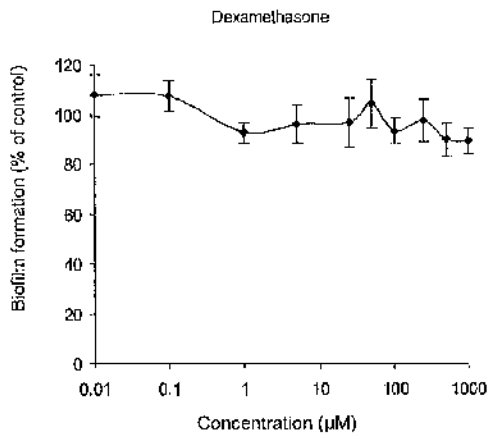
Effect of steroids on biofilm formation by *C. albicans* GDH 2346

Biofilm formation, as measured by XTT reduction, is expressed as a percentage of that of control biofilms incubated in the absence of steroids. Steroids were added at time zero of biofilm formation. Most results are means ± SEM from at least two independent experiments with a total of seven or more replicates. The mean (± SEM) control value (A_{492}) for *C. albicans* GDH 2346 ranged from 1.943 ± 0.125 to 2.241 ± 0.235.


Progesterone
Biofilm formation (% of control)
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Concentration (µM)


Corticosterone
Biofilm formation (% of control)
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Concentration (µM)


Hydrocortisone
Biofilm formation (% of control)
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Concentration (µM)


Dexamethasone
Biofilm formation (% of control)
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Concentration (µM)

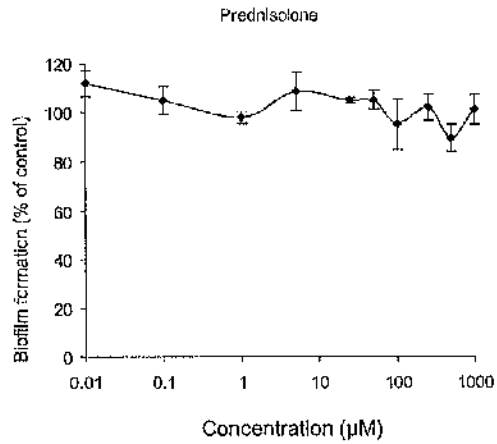

Prednisolone
Biofilm formation (% of control)
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
Concentration (µM)

Table 22. Effect of steroids on germ-tube formation by *C. albicans* GDH 2346

Steroid	Germ-tube formation (%) [a] after 5h in				
	0.01 µM	0.1 µM	1 µM	100 µM	1000 µM
Progesterone	85 ± 7	75 ± 5	66 ± 8 [d]	67 ± 5 [c]	50 ± 8 [c]
Corticosterone	111 ± 5	108 ± 6	92 ± 6	61 ± 5 [c]	35 ± 5 [b]
Dexamethasone	100 ± 5	87 ± 5	75 ± 4 [d]	80 ± 5 [d]	64 ± 9 [d]
Prednisolone	92 ± 5	82 ± 5	89 ± 8	44 ± 5 [b]	36 ± 5 [b]
Hydrocortisone	83 ± 4	90 ± 5	94 ± 5	79 ± 4 [d]	52 ± 5 [c]
Estradiol	101 ± 5	91 ± 4	85 ± 3	ND	ND

[a] Steroids were present at the concentrations indicated during germ-tube formation in 10 mM proline+2.5 mM N-acetylglucosamine buffered with 50 mM potassium phosphate (pH 6.5) for 5h. Germ-tube formation is expressed as a percentage of that of controls incubated in the absence of steroids. Results are means ± SEM from one experiment carried out in triplicate. Mean (± SEM) value for controls was 68 ± 8 /100 cells counted.

[b] Value significantly different at $P < 0.001$ from that of control.

[c] Value significantly different at $P < 0.01$ from that of control.

[d] Value significantly different at $P < 0.05$ for that of control.

ND, not determined.

DISCUSSION

1. Biofilm development and germ-tube formation in *C. albicans*

1.1 Biofilm formation using different *C. albicans* strains

Tetrazolium salts such as XTT have been widely used in cell biology for measuring the metabolic activity and viability of cells ranging from mammalian to microbial ones. In this study, biofilm formation was measured using XTT reduction as described earlier by Baillie and Douglas (1999a). However, their method was modified by adding 1% glucose to enhance the XTT reduction. Since the XTT solution was prepared in PBS instead of YNB, an exogenous energy source such as glucose is required to allow the reduction of XTT by mitochondrial dehydrogenases. Thus, there was a correlation between XTT reduction and the glucose concentration used, as can be seen in Fig. 11.

Comparison of biofilm formation by six *C. albicans* mutants with altered colony morphology and different responses to farnesol showed no significant differences as compared with the wild-type strain. These mutant strains were provided by Jacob M. Hornby (School of Biological Sciences, University of Nebraska). He and his colleagues have recently shown that 95.9% of such mutant strains fully or partially reverted to wild type morphology when grown on yeast malt agar plates supplemented with farnesol (Jensen *et al.*, 2006). In this study, the mutants were used in experiments to determine the effect of farnesol on biofilm metabolic activity and structure; the results will be discussed in Section 2.1.

Other *C. albicans* mutants were also used in this study. Biofilm formation by *C. albicans* strains defective in *MDS3*, or *MDS3* and *RIM101*, was significantly less than that of the wild type. On the other hand, a *C. albicans* mutant defective in *RIM101* only produced more biofilm than the wild type. It has been shown that *C. albicans mds3* null mutants are defective in hyphal formation in alkaline media at pH 8; however, such mutants can produce germ tubes in 5% serum (Davis *et al.*, 2002). Richard *et al.* (2005) investigated *C. albicans* biofilm-defective mutants, including *C. albicans mds3* null mutants, and showed that *C. albicans mds3* null mutants do not produce substantial biofilms on silicone squares but grow as rapidly as the wild-type strain in planktonic cultures. Moreover, reconstitution of a wild-type allele for each insertion-bearing gene restored a biofilm formation ability comparable to that of the wild type (Richard *et al.*, 2005). Other studies demonstrated that mutants of *C. albicans* defective in *RIM101* have a significant reduction in virulence (Davis *et al.*, 2000), but that the *RIM101* gene is not required for biofilm formation (Richard *et al.*, 2005). This last observation was confirmed in the present study which showed that a *C. albicans* mutant defective in *RIM101* only, produced slightly more biofilm than the wild-type strain. Another important finding in this study was that the two major signalling pathways controlling hyphal formation (the efg1 and cph1 pathways) are not essential for biofilm formation *per se*, since single or double *efg1/efg1* and *cph1/cph1* mutants produced substantial yeast-only biofilms. Overall, the role of *MDS3*, *RIM101*, *EFG1* and *CPH1* in biofilm formation of *C. albicans* remains unclear.

1.2 Germ-tube formation in *C. albicans*

C. albicans can form germ tubes in the presence of different inducers. In this study, two-germ tube inducers were investigated individually or in combination, and with static incubation or with gentle shaking, to determine the optimum germination response of *C. albicans*. Proline and N-acetylglucosamine stimulated germ-tube formation to the same extent and maximal enhancement of germ-tube formation was observed using a combination of proline and N-acetylglucosamine with gentle shaking. This observation is consistent with a previous report which showed that germ-tube formation in the presence of proline and N-acetylglucosamine was greater than that in serum (Hornby *et al.*, 2001). However, serum has been described as the 'magic potion' for germ-tube formation in *C. albicans* (Ernst, 2000) and still remains as a standard method of identifying *C. albicans* in most clinical laboratories (Kim *et al.*, 2002a; Hudson *et al.*, 2004).

The quorum sensing molecule, farnesol, is known to inhibit germ-tube formation in *C. albicans*. In this study, the optimal conditions determined for germ-tube formation were used to evaluate the effect of farnesol. The results showed that farnesol completely blocked germ-tube formation in the presence of proline and N-acetylglucosamine. These findings correspond with those of Hornby *et al.* (2001) who reported that farnesol prevents mycelial development in assays using three chemically distinct triggers for germ-tube formation: L-proline, N-acetylglucosamine, and serum. Moreover, it was shown that in serum (2 to 20%), up to 250μM farnesol was required to block germ-tube formation when added at any time up to 30 min after inoculation; farnesol did not block

germination when it was added after 90 min (Mosel *et al.*, 2005). This appears to be similar to the effect of farnesol on biofilm formation, where farnesol has to be added at an early stage to show an inhibitory effect (Ramage *et al.*, 2002a; Fig. 19).

Germ-tube formation was also inhibited by propranolol, but the inhibitory effect of propranolol was less than that of farnesol and germ-tube formation was not blocked at concentrations up to 1mM. The effect of propranolol has been investigated previously; it was reported that propranolol inhibition of morphological changes in *C. albicans* was due to binding with phosphatidic acid (Baker *et al.*, 2002), which is derived from phosphatidylcholine by the action of phospholipase D. Phospholipase D secretion is thought to be one pathway from multiple parallel pathways that control dimorphic behaviour in *C. albicans* (McLain and Dolan, 1997). Previous reports on phospholipase activity in *C. albicans* have focused on secreted enzymes that may facilitate tissue invasion, and so augment virulence (Ibrahim *et al.*, 1995). Recently, Dolan *et al.* (2004) confirmed that phospholipase D is required for virulence by demonstrating that a *C. albicans* mutant defective in the secretion of phospholipase D (PLD1) was less virulent *in vivo*. Moreover, this strain was not able to form hyphae when grown on solid Spider medium (Dolan *et al.*, 2004).

Farnesol activity has been described in other eukaryotic cells; evidence has been produced that farnesol causes apoptosis in tumorigenic cells. However, exogenous diacylglycerol (DAG) was able to prevent induction of apoptosis by farnesol (Taylor *et al.*, 2005). Another report showed that farnesol inhibited the

growth of *S. cerevisiae* and there was a significant loss of intracellular DAG in farnesol-treated cells; coaddition of DAG analogues to the growth medium abolished the effect of farnesol (Machida *et al.*, 1999). These observations were confirmed by the results of this study, because the effect of farnesol on germ-tube formation by *C. albicans* was reduced by adding DAG analogues such as 1-oleoyl-2-acetyl-sn-glycerol (OAG). Overall, these findings support the idea that farnesol inhibits the phospholipase D pathway in *C. albicans*, as in *S. cerevisiae* (Machida *et al.*, 1999) and other eukaryotic cells (Taylor *et al.*, 2005).

2. Quorum sensing in *C. albicans* biofilms

2.1 The role of farnesol

In the light of recent research, it became important to understand quorum sensing in the dimorphic organism *C. albicans*. The ability of *C. albicans* to undergo dramatic changes in cellular morphology is essential for virulence. Morphological changes in *C. albicans* are modulated by environmental factors such as pH, temperature, glucose levels, and inoculum size (Odds, 1988). The inoculum size effect has been studied in detail. For example, *C. albicans* is known to develop as budding yeasts when inoculated at $> 10^6$ cells/ml, and as mycelia when inoculated at $< 10^6$ cells/ml. This dramatic change results from the production of farnesol, which is continuously excreted by *C. albicans* during growth in amounts roughly proportional to the cell density (Hornby *et al.*, 2001).

In the present study, the effect of farnesol on biofilm formation was investigated by using a catheter disk model system and by scanning electron

microscopy. The results showed that 1 mM farnesol inhibited biofilm formation by 27 % when added at the beginning of the adhesion period and again at time zero of biofilm formation. This effect was less than that demonstrated in an earlier study by Ramage and colleagues (2002a) who showed that 300 μM farnesol inhibited biofilm formation by 85% when added at time zero. However, both observations confirm that farnesol inhibits biofilm formation in two different model systems (catheter disk and multiwell plate) and in two different *C. albicans* strains.

In this study, the biofilm structure of various *C. albicans* strains was investigated after growth in the presence or absence of farnesol, propranolol or OAG. Biofilms were grown in different media to determine the effect of these compounds in environments such as minimal defined conditions (YNB medium), maximal defined conditions (RPMI 1640 medium) and hyphal stimulating conditions (Hornby medium). The results showed that farnesol-treated biofilms consist of yeast cells only, compared with control biofilms which consist of a mixture of yeasts and hyphae. This result differs from previous observations. Ramage *et al.* (2002a) showed by scanning electron microscopy that cells treated with 300 μM farnesol produced sparse biofilms which were composed predominantly of yeast cells and pseudohyphae. Similarly, confocal scanning laser microscopy revealed that cells exposed to 40 μM farnesol formed poor biofilms consisting of yeast cells and pseudohyphae (Cao *et al.*, 2005). A possible explanation for the difference between this study and the other observations could be the farnesol concentration used; here, a higher farnesol concentration (1 mM) was employed to evaluate the effect on biofilm metabolic activity and to

investigate biofilm architecture. Biofilms grown in YNB, RPMI 1640 or Hornby medium in the presence of 1 mM farnesol consisted uniformly of yeast cells.

The effects of farnesol on biofilm formation were further investigated in this study by using six *C. albicans* mutants with altered colony morphology and different responses to farnesol. The results showed that farnesol at three concentrations (50 μM, 100 μM or 1 mM), added for the adhesion period only, or for adhesion and again at time zero of biofilm formation, did not significantly affect biofilm formation by these mutant strains as compared with the parental strain. However, scanning electron microscopy indicated that the farnesol responses of these strains were different. For example, the architecture of *C. albicans* H48 and H51 biofilms appeared to be unaffected by farnesol, because both farnesol-treated and untreated biofilms retained a mainly hyphal structure. Other mutant strains such as *C. albicans* H121 and H253 showed a complete response to farnesol by producing yeast-only biofilms. On the other hand, biofilms of *C. albicans* H1 contained mainly yeasts with a few hyphae which indicated a partial response to farnesol. These findings correspond with recent observations by Jensen *et al.* (2006), who characterized a collection of 1,111 *C. albicans* mutants that were altered in their colony morphology and in their response to farnesol, including those used here. They found that 95.9% of the morphological mutants responded reversibly to farnesol by changing their colony morphology when grown on yeast malt agar plates supplemented with 10, 50 or 100 μM farnesol. *C. albicans* H253 and H121 reverted to the wild-type morphology on yeast malt agar plates supplemented with farnesol; on the other hand *C. albicans* H1 had a partial farnesol response (Jensen *et al.*, 2006). A

possible explanation for *C. albicans* H48 and H51 biofilm structure, which remained unaffected by farnesol, would be that these strains are included in the remaining 4.1% of *C. albicans* mutants designated by Jensen *et al.* as farnesol non-responders.

Previous work demonstrated that quorum sensing molecules could be isolated from planktonic cell cultures. For example, supernatant recovered from planktonic cells grown in glucose-phosphate-proline medium inhibited germ-tube formation in two complementary bioassays, both assessing the percent germ-tube formation at 37°C but differing in whether or not the cells were provided with a complete growth medium during the bioassay (Hornby *et al.*, 2001). In this study, the inhibitory effect of quorum sensing molecules present in culture supernatant was investigated using two assays of germ-tube formation. In the first assay (Fig 28 and 29), supernatant recovered from biofilms grown in RPMI 1640 (in tissue culture flasks) was examined against planktonic cells also grown in RPMI 1640. In the second assay (Fig. 30), supernatants recovered from planktonic cells and biofilms grown in YNB were examined against planktonic cells grown in YNB. The results showed that all recovered supernatants contained a quorum sensing activity (ie. farnesol, and/or possibly other, similar, quorum sensing molecules) which inhibited the formation of germ tubes and hyphae by planktonic cells. Moreover, in the second assay, when the inhibitory effect of supernatants from planktonic cells and biofilms was calculated as a function of cell dry weight, it became clear that biofilms produce more quorum sensing molecules than do planktonic cells.

The finding that biofilms produce more quorum sensing molecules than planktonic cells lends weight to the suggestion that farnesol could provide a novel target for the development of antifungal drugs intended to prevent mycelial growth or biofilm production in *C. albicans*. Hornby *et al.* (2003) showed that *C. albicans* synthesizes farnesol from farnesyl pyrophosphate (an intermediate in the highly conserved sterol biosynthetic pathway); compounds that block the sterol pathway beyond farnesyl pyrophosphate, such as zaragozic acid B, caused an eightfold increase in the amount of farnesol produced by *C. albicans*. Moreover, antifungal agents such as fluconazole, ketoconazole, miconazole and clotrimazole have been found to elevate farnesol production in *C. albicans* by 10- to 45-fold, suggesting that increased farnesol production could be used to screen drugs that target the sterol biosynthetic pathway (Hornby and Nickerson, 2004). However, another report showed that anaerobically grown *C. albicans* did not produce farnesol and such cells were resistant to the highest tested levels of amphotericin B and four of the azole antifungals (Dumitru *et al.*, 2004).

A histidine kinase two-component signal pathway appears to play a role in regulating morphogenesis and virulence in *C. albicans* (Calera and Calderone, 1999). Recently, it was reported that a mutant lacking the histidine kinase protein Chk1p, but not the Sln1p or Nik1p histidine kinase, is refractory to the inhibitory effect of farnesol both in cell suspension and during the formation of a biofilm (Kruppa *et al.*, 2004). Chk1p therefore functions as a putative cytoplasmic farnesol sensor. In the present study, no significant differences were observed between biofilms formed by *C. albicans* CAF2-1 and two mutant strains up to 24h of incubation. However, at later time periods (48 to 72h), *C. albicans* Chk21

(*chk1/chk1*) showed significantly increased biofilm formation compared with *C. albicans* CAF2-1 (wild-type) and Chk23 (*chk1/CHK1*). This finding supports the conclusion that strain Chk21 is unaffected by farnesol. Biofilm formation by both *C. albicans* CAF2-1 and Chk23 would be responsive to endogenous farnesol levels and at the quorum sensing threshold reached during the later stages of biofilm formation, some of the newly formed yeast cells might be released into the medium. *In vivo*, such cell detachment would allow the organism to colonize a new, nutrient-rich substrate area (Ramage *et al.*, 2002a). With strain Chk21, however, which is unresponsive to farnesol, biofilm formation continues to increase. Recent work also indicates that cell detachment from these biofilms is significantly less than that from biofilms formed by the wild-type strain (B.P. Krom, personal communication).

Analysis of gene expression in *C. albicans* biofilms may lead to new insights into the molecular mechanism of farnesol action. In a recent study, Cao *et al.* (2005) investigated global gene expression in *C. albicans* biofilms grown in the presence or absence of farnesol using cDNA microarray analysis. A total of 274 genes were identified as responsive in farnesol-treated biofilms, with 104 genes up-regulated and 170 genes down-regulated. Several genes involved in hyphal formation were differentially expressed; the *TUP1* gene was up-regulated while the *PDE2* and *CRK1* genes were down-regulated. Some genes related to drug resistance were also differentially expressed. Interestingly, the cell surface hydrophobicity-associated gene *CSH1* was down-regulated. In further experiments Cao *et al.* demonstrated a negative correlation between farnesol concentration and cell surface hydrophobicity in farnesol-treated biofilm cells.

The authors concluded that a decrease in cell surface hydrophobicity might contribute to the inhibition of biofilm formation (Cao *et al.*, 2005).

2.2 The role of tyrosol

Tyrosol was identified in a recent study (Chen *et al.*, 2004) as an autoregulatory molecule that can play an important role in growth and morphogenesis in *C. albicans*. Supernatant recovered from high-density cultures contained tyrosol and this compound allowed highly diluted cultures to resume exponential growth without a substantial lag. The tyrosol activity was specific to the lag phase and had no effect on the exponential growth of *C. albicans*. Moreover, tyrosol accelerated the conversion of yeasts to filaments (Chen *et al.*, 2004). Previously, tyrosol had been isolated from *Candida* species (Narayanan and Rao, 1976; Cremer *et al.*, 1999) and *S. cerevisiae* (Sentheshanmuganathan and Elsden, 1958; Batrakov *et al.*, 1993) but its quorum sensing activity was not investigated. Later work showed that some fungi can interact with tyrosol. For example, a recent report demonstrated that saprobic fungi are able to remove tyrosol from a dry olive residue obtained from olive oil extraction (Sampedro *et al.*, 2004). Another study showed that tyrosol and other components produced by *Enterobacter cloacae* had antifungal activity against potato dry rot caused by *Gibberella pulicaris* and *Fusarium sambucinum* (Slininger *et al.*, 2004).

The results of this study demonstrated that tyrosol was produced by both planktonic cells and biofilms of *C. albicans*. Moreover, biofilms produced significantly more tyrosol than planktonic cells for four different *C. albicans* strains, including the morphological mutants *C. albicans* JKC 19 (*cph1/cph1*),

HLC 52 (*efg1/efg1*) and HLC 54 (*cph1/cph1 efg1/efg1*), and the wild-type strain *C. albicans* SC5314. Tyrosol was detected at a concentration of 8.7 μM in planktonic cultures of *C. albicans* SC5314 after 24h of incubation; this is a higher concentration of tyrosol than that detected with the same strain in a previous report (Chen *et al.*, 2004). These differences in tyrosol production could be due to differences in the incubation temperature used. Here, 37°C was used as it is the standard incubation temperature for planktonic cells and biofilms (Baillie and Douglas, 1999a; Baillie and Douglas, 1999b), whereas 30°C was used in the earlier study (Chen *et al.*, 2004). Another possible reason for the low values of tyrosol reported by Chen and his colleagues might be some loss of tyrosol during the sample clean-up procedure. There was no information in their report about the efficiency of the method used for extracting tyrosol. In contrast, the method developed here has been carefully evaluated and shown to have high levels of accuracy and precision (92.8 ± 0.8 % recovery of tyrosol). Overall, the results revealed a correlation between tyrosol production and cell dry weight for planktonic cells and biofilms of all strains tested. These findings are similar to those of Chen *et al.* (2004) and support their suggestion that tyrosol, like farnesol, could act as a quorum sensing molecule.

As tyrosol appears to be a positive quorum-sensing molecule, whereas farnesol is a negative quorum-sensing molecule (Hornby *et al.*, 2001; Oh *et al.*, 2001), it was of interest to investigate how the two interact during biofilm formation. The inhibitory effect of 50 μM farnesol on biofilm formation was abolished by tyrosol at concentrations of 100 to 1000 μM (Fig. 40). On the other hand, tyrosol did not counteract higher farnesol concentrations which

demonstrates the complex response of *C. albicans* to these two quorum-sensing molecules during biofilm development.

C. albicans biofilms grown on catheter disks consist of a dense network of yeasts, germ tubes, pseudohyphae, hyphae and extracellular material, as shown by scanning electron microscopy (Hawser and Douglas, 1994). Moreover, such biofilms comprise two layers: a thin, basal yeast layer and a thicker, hyphal layer. Similar results were found in this study with *C. albicans* biofilms formed on polystyrene disks obtained from flat-bottom, tissue culture flasks. Other studies have shown that *C. albicans* biofilms formed on acrylic material also have this structure (Chandra *et al.*, 2001a; Ramage *et al.*, 2001a). Taken together, all these reports indicate a uniformity of structure with *C. albicans* biofilms grown on different plastic surfaces. However, biofilms formed on cylindrical cellulose filters were quite different in appearance. For example, Baillie and Douglas (1999b) evaluated and compared biofilms produced by two wild-type strains with those formed by two morphological mutants incapable of yeast or hyphal growth; they showed that a hypha-negative mutant and both wild types produced exclusively yeast-form biofilms, whereas the yeast-negative mutant generated a dense hyphal mat on the top of the filter. The authors suggested that the structure of a *C. albicans* biofilm depends on the nature of the contact surface (Baillie and Douglas, 1999b). Recently, Kumamoto (2005) demonstrated that *C. albicans* biofilms exhibit contact-dependent cellular behaviour. Physical contact by *C. albicans* results in activation of the MAP kinase Mkc1p. A *C. albicans mkc1* null mutant grown on a polystyrene surface produced an abnormal biofilm with reduced filamentation compared with wild-type strains (Kumamoto, 2005).

The early stages of biofilm formation by *C. albicans* appear to be sensitive to the enhancing effect of tyrosol on hyphal development. It has been shown that germ-tube formation is stimulated by tyrosol (Chen *et al.*, 2004). This finding was confirmed here; scanning electron microscopy revealed that tyrosol-treated biofilms showed more hyphal development at early stages of biofilm formation as compared with untreated biofilms. However, there were no differences between mature (48-h) biofilms grown with and without tyrosol. A possible reason for this would be that tyrosol has a time-dependent effect similar to that of farnesol, where only the early stages of germ-tube formation and biofilm development are responsive (Ramage *et al.*, 2002a; Mosel *et al.*, 2005). Another possible reason might be that during the early stages of biofilm formation, minimal endogenous tyrosol is produced (up to 0.1 μM at 5h), whereas later there is a rapid increase to a sufficient concentration. This could explain why mature biofilms (48h) do not respond to exogenous tyrosol.

In general, the discovery of the *C. albicans* quorum-sensing molecules, farnesol and tyrosol, could lead to the development of novel signal interference approaches for controlling *Candida* infection and biofilm formation. Further investigation of these quorum-sensing systems should produce more information about the variety of signals that are employed by *Candida*, and about their biosynthesis, as well as how the information encoded in these chemical signals is processed.

3. Effect of COX inhibitors on *Candida* biofilms

Microbial biofilms are notoriously resistant to antimicrobial agents of various types, including biocides, antibiotics and antiseptics (Mah and O'Toole, 2001; Gilbert *et al.*, 2002). For example, clinically important antifungal agents such as amphotericin B, fluconazole and itraconazole are all much less active against *C. albicans* biofilms than against planktonic cells (Hawser and Douglas, 1995). Drug resistance has been demonstrated for *Candida* biofilms growing on surfaces such as cellulose (Baillie and Douglas, 1998b; Baillie and Douglas, 1999a), polystyrene (Ramage *et al.*, 2001a; Ramage *et al.*, 2002a), and denture acrylic (Chandra *et al.*, 2001b), as well as polyvinyl chloride (Hawser and Douglas, 1995). Recently, however, it has been reported that the newly introduced antifungal agent, caspofungin, is active against *C. albicans* biofilms *in vitro* (Bachmann *et al.*, 2002; Kuhn *et al.*, 2002b; Ramage *et al.*, 2002a) . The results presented here show that aspirin, one of the oldest and most widely used anti-inflammatory drugs, also dramatically decreases biofilm formation by *C. albicans*. Moreover, some aspirin concentrations (50-200 μM) producing significant levels of antibiofilm activity *in vitro* fall within the range of those frequently achieved by therapeutic doses of aspirin in humans (Xu *et al.*, 1999; Wu, 2000). Other nonsteroidal anti-inflammatory drugs, particularly etodolac and diclofenac, also inhibited biofilm formation to a significant but lesser extent.

Aspirin (acetylsalicylic acid) has a short half-life in circulating blood (about 20 min) and is rapidly deacetylated to form salicylic acid *in vivo* (Wu, 2000). Sodium salicylate and related compounds such as aspirin are known to

have a variety of effects on microorganisms. Growth of certain bacteria in the presence of salicylate can induce multiple resistance to antibiotics. Paradoxically, it can also reduce resistance to some antibiotics (Price *et al.*, 2000). *Escherichia coli*, for example, exhibits increased resistance to chloramphenicol, ampicillin, nalidixic acid and tetracycline after such treatment (Rosner, 1985). On the other hand, *E. coli* cells grown in the presence of salicylate are more sensitive to aminoglycosides (Aumercier *et al.*, 1990). The activity of antifungal agents can also be affected by salicylate. A combination of fluconazole with either sodium salicylate or ibuprofen results in synergistic activity against *C. albicans* (Scott *et al.*, 1995; Pina-Vaz *et al.*, 2000).

Sodium salicylate inhibits biofilm formation by *P. aeruginosa* and *S. epidermidis* on contact lenses and medical polymers such as polyethylene and polystyrene. Bacterial adhesion also decreases in a dose-dependent manner (Farber *et al.*, 1995). Some strains of *S. epidermidis* secrete mucoid extracellular polymers (polysaccharides, proteins and teichoic acid) that promote biofilm formation and become important components of the biofilm matrix. Salicylate can inhibit the production of some of these components by as much as 95% (Farber and Wolff, 1992; Muller *et al.*, 1998). It has been suggested that coating catheters with salicylate, or incorporating it into contact lens solutions, might decrease the incidence of some device-related infections (Farber and Wolff, 1992; Farber *et al.*, 1995).

In mammalian systems, all of the classic NSAIDs such as aspirin, diclofenac and indomethacin inhibit both the COX-1 and COX-2 isoenzymes

(Dannhardt and Kiefer, 2001). The cyclooxygenase active site is created by a long hydrophobic channel that is also the site of drug binding. Aspirin is the only known NSAID that covalently binds to a serine residue and inhibits COX-1 more than COX-2 (Dannhardt and Kiefer, 2001). In this study, seven out of the nine COX inhibitors tested decreased biofilm formation by *C. albicans*, with aspirin, diclofenac and etodolac, a preferential COX-2 inhibitor, producing the greatest effects. Aspirin and etodolac also significantly reduced the viability of biofilm cells. Indeed, aspirin appears to show an even greater effect on viability than on biofilm formation; presumably aspirin-treated biofilm cells are largely incapable of cell division but still retain some metabolic activity, including that measured in the XTT assay. Nothing is known of cyclooxygenase enzymes in *C. albicans*, but the complex effects of COX inhibitors on viability, biofilm formation and morphogenesis demonstrated here, and those on prostaglandin production described earlier (Noverr *et al.*, 2001), suggest the existence of one or more cyclooxygenase-dependent pathways in this organism. At relatively high concentrations (1 mM), however, the toxicity of aspirin for *C. albicans* is such that the drug probably has multiple, non-specific effects on fungal physiology.

Prostaglandin secretion by *C. albicans* is likely to be an important factor in the pathogenesis of many infections. In experiments with mammalian cells, purified fungal prostaglandin down-modulated chemokine production, tumour necrosis factor alpha production, and splenocyte proliferation while up-regulating interleukin 10 production (Noverr *et al.*, 2001). Its role in fungal biology is less clear. Synthetic PGE_2 enhanced biofilm formation and germ-tube formation by *C. albicans*, whereas COX inhibitors adversely affected both processes. Moreover,

exposure to some COX inhibitors (indomethacin and etodolac) produced biofilms that consisted almost entirely of yeast cells. Recently, farnesol has been identified as an extracellular quorum-sensing molecule in *C. albicans* that prevents mycelial development (Hornby *et al.*, 2001) and inhibits biofilm formation (Ramage *et al.*, 2002a). Fungal prostaglandin(s) may represent signalling molecules of a similar type. 3(R)-Hydroxyoxylipins which, like prostaglandins, are derived from arachidonic acid, have also been identified in *C. albicans* (Deva *et al.*, 2001). The synthesis of these compounds appears to take place in hyphae but not yeast cells, and is suppressed by aspirin (Deva *et al.*, 2000; Deva *et al.*, 2001). It is not yet clear whether 3(R)-hydroxyoxylipins are excreted from the cell or how they relate to fungal prostaglandins. However, it seems likely that a range of biologically active lipid molecules are involved in the regulation of biofilm formation, morphogenesis and other major physiological processes in *C. albicans*.

4. Combined effect of COX inhibitors and antifungal agents on *Candida*

The use of combination therapy may be one approach to improve the treatment of infectious diseases, to reduce dose toxicity and to enhance efficacy and safety (Baddley and Pappas, 2005). In invasive mycoses such a strategy has been used *in vivo* to promote the effectiveness of each antifungal agent (Nivoix *et al.*, 2006). Many drugs have been investigated, including antifungal and non-antimicrobial agents such as COX inhibitors (Scott *et al.*, 1995; Pina-Vaz *et al.*, 2005). In this study, aspirin was found to inhibit biofilm formation of *C. albicans* (Table 7, 10 and 11). Consequently, the combined effect of aspirin or other COX

inhibitors and amphotericin B was investigated with both planktonic cultures and biofilms. Using NCCLS methodology, it was shown that aspirin did not affect planktonic growth of *C. albicans* as evaluated visually. To further investigate this aspect, the effects of aspirin and other COX inhibitors were determined with different media and buffers, and at different pH values. The activity of these COX inhibitors on planktonic growth was assessed by optical density and viability measurements.

The results showed that aspirin did not affect planktonic cell optical density in buffered YNB or RPMI 1640 (using the NCCLS protocol). However, COX inhibitors reduced the optical density and viability in YNB by up to 42% and 95%, respectively (Fig. 48), but in buffered YNB by only 15% and 10%, respectively (Fig. 49). Similar results were obtained for aspirin with planktonic cells grown in different buffered media (Fig. 50). These experiments eliminated possible interference by media composition or buffer type on COX inhibitor activity. Thus, it appears that aspirin activity is pH-dependent (Fig. 51). For biofilm formation (as measured by metabolic activity and viability) similar results was found. Taken together, all these results demonstrated that the effect of aspirin was pH-dependent. Growth experiments using buffered medium showed that the inhibition of biofilm development was minimal (35% inhibition) at pH 7 and maximal (>85% inhibition) at low pH values.

The effect of pH on aspirin activity appears similar to that of pH on fluconazole activity described in earlier reports. For example, the interpretation of end points in azole antifungal drug susceptibility testing is problematic, in part

due to incomplete growth inhibition of *Candida* species. This can cause the MICs (in RPMI 1640, pH 7) of fluconazole for *Candida* isolates to be low (<1 μg/ml) after 24 h of growth but much higher (>64 μg/ml) after 48 h. Such isolates are considered to be resistant to fluconazole by the NCCLS protocol (Marr *et al.*, 1999). However, growing evidence suggests that these *Candida* strains are susceptible *in vivo*. For example, Rex *et al.* (1998) showed that mice infected with a fluconazole-resistant strain of *C. albicans* (MIC >64 μg/ml) responded to fluconazole administration in a manner similar to that of a susceptible strain. Further investigations by Marr *et al* (1999) demonstrated that 65% of *C. albicans* strains showed a higher MIC in RPMI 1640 (pH 7) than in unbuffered YNB or RPMI 1640 (pH 4.5) where the MIC was 32 to 64-fold lower.

Another study demonstrated that the activity of flurbiprofen (a COX inhibitor) required to inhibit the growth of some fungi including *C. albicans* depends on the pH value of the growth medium; a lower MIC_{50} was attained at pH 4 (Chowdhury *et al.*, 2003). Other reports confirm that pH values appear to play an important role in the *in vitro* activity of many antifungal agents. For example, Te Dorsthorst *et al.* (2004) investigated 21 *Aspergillus* isolates by testing *in vitro* susceptibilities against three antifungal agents in RPMI 1640 and YNB at pH 5.0 and 7.0. They showed that the MICs of amphotericin B and itraconazole were higher, while those of flucytosine were lower, at pH 5.0 than at pH 7.0. Later studies by the same workers confirmed that the *in vitro* activities of amphotericin B and flucytosine against yeast and mould isolates were pH-dependent. The *in vitro* activity of amphotericin B decreased when the pH was lowered, while the *in vitro* activity of flucytosine increased (Te Dorsthorst *et al.*, 2005). Both reports

suggested that the poor correlation between *in vitro* results and clinical outcome could be due to a difference between the pH of the *in vitro* susceptibility test and that at the site of infection.

Despite the effect of pH on COX inhibitor activity, a recent report showed that resistance to azole antifungal agents, particularly fluconazole, which was related to efflux pumps exporting drugs, could be reversed by exposure of *C. albicans* to fluconazole in the presence of ibuprofen; this reduced resistance to fluconazole in 68% of the *C. albicans* strains tested (fluconazole-resistant strains) (Pina-Vaz *et al.*, 2005). Drug export via efflux pumps has been described as a mechanism of resistance to azoles for *C. albicans* grown in planktonic cultures (Clark *et al.*, 1996), as well as in biofilms (Ramage *et al.*, 2002c; Mukherjee *et al.*, 2003).

A synergistic effect has also been shown with sodium salicylate combined with fluconazole against *C. albicans in vitro*, and it was suggested that sodium salicylate could be useful a chemotherapeutic agent for the management of infections due to fluconazole-resistant *C. albicans* strains (Yucesoy *et al.*, 2000). Furthermore, salicylic acid completely inhibited the growth of biofilms of *P. aeruginosa*, *Haemophilus influenzae*, *S. epidermidis* and *Streptococcus pneumoniae* on contact lenses as determined by viable counts (Bandara *et al.*, 2004).

C. albicans and *C. glabrata* are known to be the organisms responsible for most cases of vaginal *Candida* infection (Fidel, 2004; Fidel, 2005; Goswami *et al.*, 2006). In addition, *C. glabrata* is recognized to have a naturally reduced

susceptibility to azole antifungal agents, particularly fluconazole (Sobel, 1998; Fidel *et al.*, 1999). Aspirin activity was therefore investigated in this study at pH 4.2 in VS medium, and YNB buffered with acetic acid. The results showed that aspirin in this acidic environment inhibited the metabolic activity of mature biofilms of *C. albicans* and *C. glabrata* more than did fluconazole. Moreover, this inhibitory effect was confirmed by examining the viability of mature biofilms treated with aspirin. These differences between aspirin and fluconazole activity against mature biofilms grown in an acidic environment could be due to the limited activity of fluconazole (fungistatic) in a narrow pH range. For instance, Moosa *et al* (2004) evaluated the effect of fluconazole on *C. albicans* (planktonic cultures) under *in vitro* conditions resembling the vaginal microenvironment. They observed that fluconazole was fungicidal for *C. albicans* in VS medium, but not in other media at the same pH, 4.2. Subsequently, the components of VS medium were analysed and acetic acid, present at 17 mM, a concentration achieved in the vagina, was identified as the component responsible for the synergistic, fungicidal effect (Moosa *et al.*, 2004).

Overall, the results of this study demonstrate that aspirin possesses potent antibiofilm activity *in vitro* and could be useful in combined therapy with conventional antifungal agents for the management of some biofilm-associated infections in acidic environments. Several organs in the body have an acidic environment, including the vagina (Thinkhamrop *et al.*, 1999), stomach and collecting ducts of the kidney (Goenka *et al.*, 1996).

5. Prostaglandin production in planktonic cells and biofilms of *C. albicans*

Throughout the entire growth period (48h) for planktonic cultures and biofilms there was little correlation between prostaglandin concentration and cell density, suggesting that prostaglandin does not function as a quorum sensing molecule. During the process of quorum sensing, signal molecules accumulate in cultures and at a threshold population density (quorum) interact with cellular receptors that control expression of specific target genes. In *C. albicans* two signal molecules have been identified: farnesol, which acts as a negative signal and inhibits the formation of hyphae (Hornby *et al.*, 2001), and tyrosol which acts as a positive signal and promotes hyphal formation (Chen *et al.*, 2004). Like prostaglandins, both of these molecules are relatively hydrophobic and of low molecular mass.

The results of this study clearly showed that biofilms produce more prostaglandins than planktonic cells. *In vivo*, such a localized concentration of prostaglandin could significantly enhance fungal colonization and pathogenesis. Experiments with mammalian cells have already shown that purified *C. albicans* prostaglandin down-modulates chemokine production, tumor necrosis factor alpha production, and splenocyte proliferation, but up-regulates interleukin 10 production (Noverr *et al.*, 2003). Pathogen-host interactions of this type could contribute to the persistence of *Candida* infections like chronic vaginitis which is now thought to be biofilm-associated (Domingue *et al.*, 1991; Costerton *et al.*, 2003).

Previous experiments demonstrated that aspirin, diclofenac and etodolac at a concentration of 1mM inhibited the growth of *Candida* biofilms (Table 7). At a relatively at low concentration (50 μM) aspirin showed a similar effect (Fig. 44). Therefore, as expected 50 μM aspirin, diclofenac and etodolac reduced prostaglandin production by both biofilms and planktonic cells. Taken together with the earlier findings, these results demonstrate a strong correlation between decreased prostaglandin levels and decreased biofilm formation following exposure to COX inhibitors, thus supporting the notion that cyclooxygenase-dependent synthesis of prostaglandins may play a role in regulating biofilm development.

Prostaglandin production was also determined in a mutant with defined defects in two filamentation pathways (*cph1/cph1 efg1/efg1*) and its wild-type strain *C. albicans* SC5314. The mutant is a *URA3/ura3* heterozygote constructed in strain CAI4, which is derived from the clinical isolate SC5314 and contains deletions of both chromosomal copies of *URA3* (Lo *et al.*, 1997). In the mutant, the mitogen-activated protein (MAP) kinase and Ras-cAMP pathways, involving transcription factors Cph1 and Efg1, respectively, are blocked. The mutant strain produced substantial yeast-only biofilms as confirmed by scanning electron microscopy. This finding is in marked contrast to previous reports that such mutants fail to produce any biofilms (Lewis *et al.*, 2002; Ramage *et al.*, 2002), although in these other studies the growth medium used was RPMI-1640, not yeast nitrogen base.

Overall, the experiments with the morphological mutant demonstrate that the genetic defects of this strain did not affect its ability to secrete prostaglandin. Moreover, biofilms of this strain, consisting entirely of yeast cells, produced significantly more prostaglandin than did planktonic cells. Thus, although prostaglandin from either fungal or mammalian sources can promote germ-tube formation in *C. albicans* (Kalo-Klein and Witkin, 1990; Noverr *et al.*, 2001), its exact role in fungal morphogenesis and biofilm development is obviously complex and remains unclear.

6. Biofilm formation in the presence of steroids

Hormones are important biochemical signalling molecules that regulate can control physiological events in multicellular organisms; however, it is now clear that analogous hormones or pheromones can act as regulatory messengers between unicellular organisms such as yeasts (Gooday and Adams, 1993). Additionally, several investigations have been reported concerning the influence of mammalian hormones such as steroids on the pathogenic *C. albicans*. For instance, steroid compounds such as hydrocortisone, corticosterone, prednisolone and dexamethasone have been shown to inhibit the activity of neutrophils against *C. albicans*, but did not affect the growth of fungus (Nohmi *et al.*, 1994; Nohmi *et al.*, 1995).

Estrogen was found to reduce the ability of vaginal epithelial cells to inhibit the growth of *C. albicans* (Fidel *et al.*, 2000). Moreover, the estrogen hormonal status of rats affected vaginal infection with *C. albicans*. For example, Kinsman and Collard (1986) observed in an *in vivo* animal model that four hours after

infection, viable counts were higher and germ tubes were longer in those animals in estrous than in other animals (Kinsman and Collard, 1986). Subsequent studies suggested that hormone interaction with *C. albicans* could be affected by surface hydrophobicity. For example, Braun (1994) demonstrated that corticosterone incorporation by *C. albicans* is dependent on the hydrophobicity of the cell surface, and that hydrophobic yeast cells and hyphae incorporated significantly more [^{3}H] corticosterone than did hydrophilic forms of *C. albicans* .

There has also been some controversy about the influence of hormones administered in combination with antifungal agents for the treatment of *Candida* corneal infections. Many studies have demonstrated that treatment with corticosteroids in combination with antifungal agents has a negative effect on fungal infections (Stover *et al.*, 1983; Stern and Buttross, 1991). However, Krishnamurthy *et al* (1998) examined the expression of *CDR1* (*Candida* drug resistance gene) in the presence of estradiol and progesterone, and showed that estradiol and progesterone were both able to enhance *CDR1* expression levels about 4.5-fold (Krishnamurthy *et al.*, 1998). On the other hand, Schreiber *et al.* (2003) showed that the administration of additional prednisolone was clearly the most effective treatment to reduce *C. albicans* corneal infection in a rabbit model (Schreiber *et al.*, 2003).

In the light of this information, it was of interest to investigate the role of these hormones against *C. albicans* biofilms and germ-tube formation. Overall, the results of these experiments indicate that biofilm metabolic activity was not affected by steroids. This is consistent with previous observations which showed

that steroid compounds did not have a direct effect on growth of *C. albicans* planktonic cells (Nohmi *et al.*, 1994). However, steroids could play an important role in *Candida* virulence, because microorganisms possess the ability to recognize hormones within the host and utilize them to adapt to their surroundings. Steroids may therefore serve as environmental factors which could induce *Candida* virulence. Recent data confirm that *C. albicans* can become pathogenic, producing signs and symptoms of disease in certain conditions, and that steroid therapy was one of the most important predisposing factors (Cheng *et al.*, 2005) .

Although there are no published data on the interaction between hormones and microbial biofilms, Roberts *et al.* (2002; 2005) reported evidence for a direct role of stress hormones upon growth of the oral bacteria commonly found within biofilms of subgingival plaque (below the gum margin). They demonstrated that there was a broad variation in the growth responses of 43 microorganisms to noradrenaline or adrenaline, with half of the species showing significant growth enhancement. The authors concluded that stress hormones might directly modulate the growth and composition of the subgingival biofilm (Roberts *et al.*, 2002; Roberts *et al.*, 2005). Biofilm formation by *C. albicans*, as tested in this study, was not similarly affected by steroids although only five steroid compounds were investigated. This could be an area worthy of further, more detailed, experimentation.

7. Overall conclusions

Community behaviour by *C. albicans* was recognized recently when farnesol and tyrosol were identified as quorum sensing molecules. The data presented in this thesis demonstrate that farnesol inhibits hyphal formation and tyrosol promotes hyphal formation in both planktonic cells and biofilms. Specific signalling pathways mediate different environmental signals to regulate morphogenesis. Two of the best studied pathways are the mitogen-activated protein (MAP) kinase and Ras-cAMP pathways, involving transcription factors Cph1 and Efg1, respectively. Signals include chemical parameters (such as pH), morphogens (such as serum), nutrient starvation and physical parameters. Operation of these pathways results in activation or repression of hypha-specific genes in response to an environmental signal. For example, farnesol in the surrounding medium decreases the expression of a hypha-specific wall protein. Other signal molecules produced by *C. albicans* include prostaglandins. Unlike farnesol and tyrosol, prostaglandins are not quorum-sensing molecules but they act as signals to stimulate hyphal production. Figure 62 illustrates possible interactions between these various signalling pathways during morphogenesis and biofilm formation in *C. albicans*.

Figure 62

Operation of different signalling pathways during morphogenesis and biofilm formation in *C. albicans*

Hyphae
Stimulation
Celecoxib
Etodolac
Cox-2 inhibitors
Decreases *HWP1* expression
(Hypha-specific wall protein)
Farnesol
Cph1
Hypha-specific genes
Efg1
Tyrosol
MAP
kinase
cascade
cAMP
Signal
Arachidonic acid
COX-2
COX-1
Prostaglandin synthases
Prostaglandins
Cox-1 inhibitors
Aspirin
Diclofenac
Yeast

REFERENCES

Adam, B., Baillie, G. S. & Douglas, L. J. (2002). Mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*. *Journal of Medical Microbiology* **51**, 344-349.

Alaci, S., Larcher, C., Ebenbichler, C., Prodinger, W. M., Janatova, J. & Dierich, M. P. (1993). Isolation and biochemical characterization of the Ic3b receptor of *Candida albicans*. *Infection and Immunity* **61**, 1395-1399.

Al-Fattani, M. A. & Douglas, L. J. (2004). Penetration of *Candida* biofilms by antifungal agents. *Antimicrobial Agents and Chemotherapy* **48**, 4073-4073.

Andes, D., Nett, J., Oschel, P., Albrecht, R., Marchillo, K. & Pitula, A. (2004). Development and characterization of an *in vivo* central venous catheter *Candida albicans* biofilm model. *Infection and Immunity* **72**, 6023-6031.

Arai, R., Sugita, T. & Nishikawa, A. (2005). Reassessment of the *in vitro* synergistic effect of fluconazole with the non-steroidal anti-inflammatory agent ibuprofen against *Candida albicans*. *Mycoses* **48**, 38-41.

Aumercier, M., Murray, D. M. & Rosner, J. L. (1990). Potentiation of susceptibility to aminoglycosides by salicylate in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* **34**, 786-791.

Bachmann, S. P., VandeWalle, K., Ramage, G., Patterson, T. F., Wickes, B. L., Graybill, J. R. & Lopez-Ribot, J. L. (2002). *In vitro* activity of caspofungin against *Candida albicans* biofilms. *Antimicrobial Agents and Chemotherapy* **46**, 3591-3596.

Baddley, J. W. & Pappas, P. G. (2005). Antifungal combination therapy - Clinical potential. *Drugs* **65**, 1461-1480.

Baillie, G. S. & Douglas, L. J. (1998a). Effect of growth rate on resistance of *Candida albicans* biofilms to antifungal agents. *Antimicrobial Agents and Chemotherapy* **42**, 1900-1905.

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

Baillie, G. S. & Douglas, L. J. (1999a). *Candida* biofilms and their susceptibility to antifungal agents. In *Methods in Enzymology*, pp. 644-656.

Baillie, G. S. & Douglas, L. J. (1999b). Role of dimorphism in the development of *Candida albicans* biofilms. *Journal of Medical Microbiology* **48**, 671-679.

Baillie, G. S. & Douglas, L. J. (2000). Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. *Journal of Antimicrobial Chemotherapy* **46**, 397-403.

Baker, C. A., Desrosiers, K. & Dolan, J. W. (2002). Propranolol inhibits hyphal development in *Candida albicans*. *Antimicrobial Agents and Chemotherapy* **46**, 3617-3620.

Bandara, B. M. K., Sankaridurg, P. R. & Willcox, M. D. P. (2004). Non-steroidal anti inflammatory agents decrease bacterial colonisation of contact lenses and prevent adhesion to human corneal epithelial cells. *Current Eye Research* **29**, 245-251.

Banuett, F. (1998). Signalling in the yeasts: An informational cascade with links to the filamentous fungi. *Microbiology and Molecular Biology Reviews* **62**, 249-274.

Bates, S., Hughes, H. B., Munro, C. A., Thomas, W. P. H., MacCallum, D. M., Bertram, G., Atrih, A., Ferguson, M. A. J., Brown, A. J. P., Odds, F. C. & Gow, N. A. R. (2006). Outer chain N-glycans are required for cell wall integrity and virulence of Candida albicans. *Journal of Biological Chemistry* **281**, 90-98.

Batrakov, S. G., Elregistan, G. I., Pridachina, N. N., Nenashev, V. A., Kozlova, A. N., Gryaznova, M. N. & Zolotareva, I. N. (1993). Tyrosol as autoregulatory factor D(1) in the yeast *Saccharomyces cerevisiae*. *Microbiology* **62**, 387-390.

Berlanga, M. & Vinas, M. (2000). Salicylate induction of phenotypic resistance to quinolones in *Serratia marcescens*. *Journal of Antimicrobial Chemotherapy* **46**, 279-282.

Bodey, G. P. (1993). *CANDIDIASIS: Pathogenesis, diagnosis and treatment*, Second Edition edn: Raven Press, New York.

Bodey, G. P., Mardani, M., Hanna, H. A., Boktour, M., Abbas, J., Girgawy, E., Hachem, R. Y., Kontoyiannis, D. P. & Raad, I. I. (2002). The epidemiology of *Candida glabrata* and *Candida albicans* fungemia in immunocompromised patients with cancer. *American Journal of Medicine* **112**, 380-385.

Braun, P. C. (1994). Surface hydrophobicity enhances corticosterone incorporation in *Candida albicans*. *Infection and Immunity* **62**, 4087-4090.

Brown, A. J. P. & Gow, N. A. R. (1999). Regulatory networks controlling *Candida albicans* morphogenesis. *Trends in Microbiology* 7, 333-338.

Burke, Y. D., Ayoubi, A. S., Werner, S. R., McFarland, B. C., Heilman, D. K., Ruggeri, B. A. & Crowell, P. L. (2002). Effects of the isoprenoids perillyl alcohol and farnesol on apoptosis biomarkers in pancreatic cancer chemoprevention. *Anticancer Research* **22**, 3127-3134.

Buurman, E. T., Westwater, C., Hube, B., Brown, A. J. P., Odds, F. C. & Gow, N. A. R. (1998). Molecular analysis of CaMnt1p, a mannosyl transferase important for adhesion and virulence of *Candida albicans*. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 7670-7675.

Calderone, R. (2002). *Candida and Candidiasis*. Washington, DC: American Society for Microbiology.

Calera, J. A. & Calderone, R. (1999). Histidine kinase, two-component signal transduction proteins of *Candida albicans* and the pathogenesis of candidosis. *Mycoses* **42**, 49-53.

Calera, J. A., Zhao, X. J., De Bernardis, F., Sheridan, M. & Calderone, R. (1999). Avirulence of *Candida albicans* CaHK1 mutants in a murine model of hematogenously disseminated candidiasis. *Infection and Immunity* **67**, 4280-4284.

Cameron, B. J. & Douglas, L. J. (1996). Blood group glycolipids as epithelial cell receptors for *Candida albicans*. *Infection and Immunity* **64**, 891-896.

Canuto, M. M. & Rodero, F. G. (2002). Antifungal drug resistance to azoles and polyenes. *Lancet Infectious Diseases* **2**, 550-563.

Cao, Y. Y., Cao, Y. B., Xu, Z., Ying, K., Li, Y., Xie, Y., Zhu, Z. Y., Chen, W. S. & Jiang, Y. Y. (2005). cDNA microarray analysis of differential gene expression in *Candida albicans* biofilm exposed to farnesol. *Antimicrobial Agents and Chemotherapy* **49**, 584-589.

Chandra, J., Kuhn, D. M., Mukherjee, P. K., Hoyer, L. L., McCormick, T. & Ghannoum, M. A. (2001a). Biofilm formation by the fungal pathogen *Candida albicans*: Development, architecture, and drug resistance. *Journal of Bacteriology* **183**, 5385-5394.

Chandra, J., Mukherjee, P. K., Leidich, S. D., Faddoul, F. F., Hoyer, L. L., Douglas, L. J. & Ghannoum, M. A. (2001b). Antifungal resistance of candidal biofilms formed on denture acrylic *in vitro*. *Journal of Dental Research* **80**, 903-908.

Charles, P. E., Dalle, F., Aube, H., Doise, J. M., Quenot, J. P., Aho, L. S., Chavanet, P. & Blettery, B. (2005). *Candida* spp. colonization significance in critically ill medical patients: a prospective study. *Intensive Care Medicine* **31**, 393-400.

Chen, H., Fujita, M., Feng, Q. H., Clardy, J. & Fink, G. R. (2004). Tyrosol is a quorum-sensing molecule in *Candida albicans*. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 5048-5052.

Cheng, M.-F., Yang, Y.-L., Yao, T.-J., Lin, C.-Y., Liu, J.-S., Tang, R.-B., Yu, K.-W., Fan, Y.-H., Hsieh, K.-S. & Ho et, a. (2005). Risk factors for fatal candidemia caused by *Candida albicans* and non-*albicans Candida* species. *BMC Infectious Diseases* **5**, 22.

Chowdhury, B., Adak, M. & Bose, S. K. (2003). Flurbiprofen, a unique non-steroidal anti-inflammatory drug with antimicrobial activity against *Trichophyton*, *Microsporum* and *Epidermophyton* species. *Letters in Applied Microbiology* **37**, 158-161.

Clark, F. S., Parkinson, T., Hitchcock, C. A. & Gow, N. A. R. (1996). Correlation between rhodamine 123 accumulation and atole sensitivity in *Candida* species: Possible role for drug efflux in drug resistance. *Antimicrobial Agents and Chemotherapy* **40**, 419-425.

Costerton, J. W. (1995). Overview of microbial biofilms. *Journal of Industrial Microbiology* **15**, 137-140.

Costerton, W., Veeh, R., Shirtliff, M., Pasmore, M., Post, C. & Ehrlich, G. (2003). The application of biofilm science to the study and control of chronic bacterial infections. *Journal of Clinical Investigation* **112**, 1466-1477.

Cremer, J., Vatou, V. & Braveny, I. (1999). 2,4-(hydroxyphenyl)-ethanol, an antioxidative agent produced by *Candida* spp., impairs neutrophilic yeast killing *in vitro*. *FEMS Microbiology Letters* **170**, 319-325.

Critchley, I. A. & Douglas, L. J. (1987a). Role of gycosides as epithelial-cell receptors for *Candida albicans*. *Journal of General Microbiology* **133**, 637-643.

Critchley, I. A. & Douglas, L. J. (1987b). Isolation and partial characterization of an adhesin from *Candida albicans*. *Journal of General Microbiology* **133**, 629-636.

Dannhardt, G. & Kiefer, W. (2001). Cyclooxygenase inhibitors - current status and future prospects. *European Journal of Medicinal Chemistry* **36**, 109-126.

Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W. & Greenberg, E. P. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **280**, 295-298.

Davis, D., Edwards, J. E., Mitchell, A. P. & Ibrahim, A. S. (2000). *Candida albicans* RIM101 pH response pathway is required for host-pathogen interactions. *Infection and Immunity* **68**, 5953-5959.

Davis, D. A., Bruno, V. M., Loza, L., Filler, S. G. & Mitchell, A. P. (2002). *Candida albicans* Mds3p, a conserved regulator of pH responses and virulence identified through insertional mutagenesis. *Genetics* **162**, 1573-1581.

Deslauriers, N., Coulombe, C., Carre, B. & Goulet, J. P. (1995). Topical application of a corticosteroid destabilizes the host-parasite relationship in an experimental-model of the oral carrier state of *Candida albicans*. *FEMS Immunology and Medical Microbiology* **11**, 45-55.

Deva, R., Ciccoli, R., Kock, L. & Nigam, S. (2001). Involvement of aspirin-sensitive oxylipins in vulvovaginal candidiasis. *FEMS Microbiology Letters* **198**, 37-43.

Deva, R., Ciccoli, R., Schewe, T., Kock, J. L. F. & Nigam, S. (2000). Arachidonic acid stimulates cell growth and forms a novel oxygenated metabolite in *Candida albicans*. *Biochimica Et Biophysica ACTA-Molecular and Cell Biology of Lipids* **1486**, 299-311.

Dolan, J. W., Bell, A. C., Hube, B., Schaller, M., Warner, T. F. & Balish, E. (2004). *Candida albicans* PLD1 activity is required for full virulence. *Medical Mycology* **42**, 439-447.

Domingue, P. A. G., Sadhu, K., Costerton, J. W., Bartlett, K. & Chow, A. W. (1991). The human vagina - normal flora considered as an in situ tissue-associated, adherent biofilm. *Genitourinary Medicine* **67**, 226-231.

Donelli, G. & Francolini, I. (2001). Efficacy of antiadhesive, antibiotic and antiseptic coatings in preventing catheter-related infections: Review. *Journal of Chemotherapy* **13**, 595-606.

Donlan, R. M. (2002). Biofilms: Microbial life on surfaces. *Emerging Infectious Diseases* **8**, 881-890.

Donlan, R. M. & Costerton, J. W. (2002). Biofilms: Survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews* **15**, 167-+.

Douglas, L. J. (2003). *Candida* biofilms and their role in infection. *Trends in Microbiology* **11**, 30-36.

Dronda, F., AlonsoSanz, M., Laguna, F., Chaves, F., MartinezSuarez, J. V., RodriguezTudela, J. L., GonzalezLopez, A. & Valencia, E. (1996). Mixed oropharyngeal candidiasis due to *Candida albicans* and non-*albicans Candida* strains in HIV-infected patients. *European Journal of Clinical Microbiology & Infectious Diseases* **15**, 446-452.

Dumitru, R., Hornby, J. M. & Nickerson, K. W. (2004). Defined anaerobic growth medium for studying *Candida albicans* basic biology and resistance to eight antifungal drugs. *Antimicrobial Agents And Chemotherapy* **48**, 2350-2354.

Duncan, R. E. & Archer, M. C. (2006). Farnesol induces thyroid hormone receptor (THR) beta 1 but inhibits THR-mediated signaling in MCF-7 human breast cancer cells. *Biochemical and Biophysical Research Communications* **343**, 239-243.

El-Azizi, M. A., Starks, S. E. & Khardori, N. (2004). Interactions of *Candida albicans* with other *Candida* spp. and bacteria in the biofilms. *Journal of Applied Microbiology* **96**, 1067-1073.

Elliott, T. S. J. & Tebbs, S. E. (1998). Prevention of central venous catheter-related infection. *Journal of Hospital Infection* **40**, 193-201.

Epstein, J. B., Gorsky, M. & Caldwell, J. (2002). Fluconazole mouthrinses for oral candidiasis in postirradiation, transplant, and other patients. *Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics* **93**, 671-675.

Ernst, J. F. (2000). Transcription factors in *Candida albicans* - environmental control of morphogenesis. *Microbiology* **146**, 1763-1774.

Farber, B. F. & Wolff, A. G. (1992). The use of nonsteroidal anti-inflammatory drugs to prevent adherence of *Staphylococcus epidermidis* to medical polymers. *Journal of Infectious Diseases* **166**, 861-865.

Farber, B. F., Hsieh, H. C., Donnenfeld, E. D., Perry, H. D., Epstein, A. & Wolff, A. (1995). A Novel antibiofilm technology for contact-lens solutions. *Ophthalmology* **102**, 831-836.

Fidel, P. L. (2004). History and new insights into host defense against vaginal candidiasis. *Trends in Microbiology* **12**, 220-227.

Fidel, P. L., Vazquez, J. A. & Sobel, J. D. (1999). *Candida glabrata*: Review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. *Clinical Microbiology Reviews* **12**, 80-96.

Fidel, P. L., Cutright, J. & Steele, C. (2000). Effects of reproductive hormones on experimental vaginal candidiasis. *Infection and Immunity* **68**, 651-657.

Fidel, P. L., Jr. (2005). Immunity in vaginal candidiasis. *Current Opinion in Infectious Diseases* **18**, 107-111.

Filler, S. G., Ibe, B. O., Luckett, P. M., Raj, J. U. & Edwards, J. E. (1991). *Candida albicans* stimulates endothelial-cell eicosanoid production. *Journal of Infectious Diseases* **164**, 928-935.

Filler, S. G., Ibe, B. O., Ibrahim, A. S., Ghannoum, M. A., Raj, J. U. & Edwards, J. E. (1994). Mechanisms by which *Candida albicans* induces endothelial-cell prostaglandin synthesis. *Infection and Immunity* **62**, 1064-1069.

Finch, R. G., Pritchard, D. I., Bycroft, B. W., Williams, P. & Stewart, G. S. A. B. (1998). Quorum sensing: A novel target for anti-infective therapy. *Journal of Antimicrobial Chemotherapy* **42**, 569-571.

Fromtling, R. A. (1988). Overview of medically important antifungal azole derivatives. *Clinical Microbiololgy Reviews* **1**, 187-217.

Fu, Y., Ibrahim, A. S., Fonzi, W., Zhou, X., Ramos, C. F. & Ghannoum, M. A. (1997). Cloning and characterization of a gene (*LIP1*) which encodes a lipase from the pathogenic yeast *Candida albicans*. *Microbiology* **143**, 331-340.

Garcia-Sanchez, S., Aubert, S., Iraqui, I., Janbon, G., Ghigo, J. M. & d'Enfert, C. (2004). *Candida albicans* biofilms: a developmental state associated with specific and stable gene expression patterns. *Eukaryotic Cell* **3**, 536-545.

Gilbert, P., Maira-Litran, T., McBain, A. J., Rickard, A. H. & Whyte, F. W. (2002). The physiology and collective recalcitrance of microbial biofilm communities. *Advances in microbial physiology* **46**, 203-256.

Goenka, M. K., Kochhar, R., Chakrabarti, A., Kumar, A., Gupta, O., Talwar, P. & Mehta, S. K. (1996). *Candida* overgrowth after treatment of duodenal ulcer. A comparison of cimetidine, famotidine, and omeprazole. *Journal of Clinical Gastroenterology* **23**, 7-10.

Gooday, G. W. & Adams, D. J. (1993). Sex-hormones and fungi. *Advances in Microbial Physiology* **34**, 69-145.

Gosden, R. G., Wade, J. C., Fraser, H. M., Sandow, J. & Faddy, M. J. (1997). Impact of congenital or experimental hypogonadotrophism on the radiation sensitivity of the mouse ovary. *Human Reproduction* **12**, 2483-2488.

Goswami, D., Goswami, R., Banerjee, U., Dadhwal, V., Miglani, S., Lattif, A. A. & Kochupillai, N. (2006). Pattern of *Candida* species isolated from patients with diabetes mellitus and vulvovaginal candidiasis and their response to single dose oral fluconazole therapy. *Journal of Infection* **52**, 111-117.

Graybill, J. R. (1992). Future-directions of antifungal chemotherapy. *Clinical Infectious Diseases* **14**, S170-S181.

Hall-Stoodley, L. & Stoodley, P. (2002). Developmental regulation of microbial biofilms. *Current Opinion in Biotechnology* **13**, 228-233.

Hall-Stoodley, L. & Stoodley, P. (2005). Biofilm formation and dispersal and the transmission of human pathogens. *Trends in Microbiology* **13**, 7-10.

Harris, S. G., Padilla, J., Koumas, L., Ray, D. & Phipps, R. P. (2002). Prostaglandins as modulators of immunity. *Trends in Immunology* **23**, 144-150.

Harrison, J. J., Turner, R. J. & Ceri, H. (2005). Persister cells, the biofilm matrix and tolerance to metal cations in biofilm and planktonic Pseudomonas aeruginosa. *Environmental Microbiology* 7, 981-994.

Hawser, S. P. & Douglas, L. J. (1994). Biofilm formation by *Candida* species on the surface of catheter materials *in vitro*. *Infection and Immunity* **62**, 915-921.

Hawser, S. P. & Douglas, L. J. (1995). Resistance of *Candida albicans* biofilms to antifungal agents *in vitro*. *Antimicrobial Agents And Chemotherapy* **39**, 2128-2131.

Hawser, S. P., Baillie, G. S. & Douglas, L. J. (1998). Production of extracellular matrix by *Candida albicans* biofilms. *Journal of Medical Microbiology* **47**, 253-256.

Haynes, K. (2001). Virulence in *Candida* species. *Trends in Microbiology* **9**, 591-596.

Hobson, R. P. (2003). The global epidemiology of invasive *Candida* infections - is the tide turning? *Journal of Hospital Infection* **55**, 159-168.

Hogan, D. A. & Kolter, R. (2002). *Pseudomonas-Candida* interactions: an ecological role for virulence factors. *Science* **296**, 2229-2232.

Hogan, D. A., Vik, A. & Kolter, R. (2004). A *Pseudomonas aeruginosa* quorum-sensing molecule influences *Candida albicans* morphology. *Molecular Microbiology* **54**, 1212-1223.

Holmes, A. R., Bandara, B. M. K. & Cannon, R. D. (2002). Saliva promotes *Candida albicans* adherence to human epithelial cells. *Journal of Dental Research* **81**, 28-32.

Honraet, K., Goetghebeur, E. & Nelis, H. J. (2005). Comparison of three assays for the quantification of *Candida* biomass in suspension and CDC reactor grown biofilms. *Journal of Microbiological Methods* **63**, 287-295.

Hoover, C. I., Jantapour, M. J., Newport, G., Agabian, N. & Fisher, S. J. (1998). Cloning and regulated expression of the *Candida albicans* phospholipase B (PLB1) gene. *FEMS Microbiology Letters* **167**, 163-169.

Hornby, J. M. & Nickerson, K. W. (2004). Enhanced production of farnesol by *Candida albicans* treated with four azoles. *Antimicrobial Agents and Chemotherapy* **48**, 2305-2307.

Hornby, J. M., Kebaara, B. W. & Nickerson, K. W. (2003). Farnesol biosynthesis in *Candida albicans*: Cellular response to sterol inhibition by zaragozic acid B. *Antimicrobial Agents and Chemotherapy* **47**, 2366-2369.

Hornby, J. M., Jacobitz-Kizzier, S. M., McNeel, D. J., Jensen, E. C., Treves, D. S. & Nickerson, K. W. (2004). Inoculum size effect in dimorphic fungi:

Extracellular control of yeast-mycelium dimorphism in *Ceratocystis ulmi*. *Applied and Environmental Microbiology* **70**, 1356-1359.

Hornby, J. M., Jensen, E. C., Lisec, A. D., Tasto, J. J., Jahnke, B., Shoemaker, R., Dussault, P. & Nickerson, K. W. (2001). Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Applied and Environmental Microbiology* **67**, 2982-2992.

Hube, B., Stehr, F., Bossenz, M., Mazur, A., Kretschmar, M. & Schafer, W. (2000). Secreted lipases of *Candida albicans*: cloning, characterisation and expression analysis of a new gene family with at least ten members. *Archives of Microbiology* **174**, 362-374.

Hudson, D. A., Sascia, O. L., Sanders, R. J., Norris, G. E., Edwards, P. J. B., Sullivan, P. A. & Farley, P. C. (2004). Identification of the dialysable serum inducer of germ-tube formation in *Candida albicans*. *Microbiology* **150**, 3041-3049.

Hunt, S. M., Hamilton, M. A., Sears, J. T., Harkin, G. & Reno, J. (2003). A computer investigation of chemically mediated detachment in bacterial biofilms. *Microbiology* **149**, 1155-1163.

Ibrahim, A. S., Mirbod, F., Filler, S. G., Banno, Y., Cole, G. T., Kitajima, Y., Edwards, J. E., Nozawa, Y. & Ghannoum, M. A. (1995). Evidence implicating phospholipase as a virulence factor of *Candida albicans*. *Infection and Immunity* **63**, 1993-1998.

Imbert, C., Rodier, M. H., Daniault, G. & Jacquemin, J. L. (2002). Influence of sub-inhibitory concentrations of conventional antifungals on metabolism of *Candida albicans* and on its adherence to polystyrene and extracellular matrix proteins. *Medical Mycology* **40**, 123-129.

Jensen, E. C., Hornby, J. M., Pagliaccetti, N. E., Wolter, C. M., Nickerson, K. W. & Atkin, A. L. (in press). Farnesol restors wild-type colony morphology to 96% of *Candida albicans* colony morphology variants recovered following treatment with mutagens. *Genome* **49**, 346-353.

Jimenez-Lucho, V., Ginsburg, V. & Krivan, H. C. (1990). *Cryptococcus neoformans*, *Candida albicans*, and gther fungi bind specifically to the glycosphingolipid lactosylceramide (Gal-Beta-1-4glc-Beta-1-1cer), a possible adhesion receptor for yeasts. *Infection and Immunity* **58**, 2085-2090.

Kalo-Klein, A. & Witkin, S. S. (1990). Prostaglandin E_2 enhances and gamma interferon inhibits germ tube formation in *Candida albicans*. *Infection and Immunity* **58**, 260-262.

Karaca, M., Bayram, A., Kocoglu, M. E., Gocmen, A. & Eksi, F. (2005). Comparison of clinical diagnosis and microbiological test results in vaginal infections. *Clinical and Experimental Obstetrics and Gynecology* **32**, 172-174.

Kim, D., Shin, W. S., Lee, K. H., Kim, K., Park, J. Y. & Koh, C. M. (2002a). Rapid differentiation of *Candida albicans* from other *Candida* species using its unique germ tube formation at 39 degrees C. *Yeast* **19**, 957-962.

Kim, S., Kim, E., Shin, D. S., Kang, H. & Oh, K. B. (2002b). Evaluation of morphogenic regulatory activity of farnesoic acid and its derivatives against *Candida albicans* dimorphism. *Bioorganic and Medicinal Chemistry Letters* **12**, 895-898.

Kinsman, O. S. & Collard, A. E. (1986). Hormonal factors in vaginal candidiasis in rats. *Infection and Immunity* **53**, 498-504.

Kjelleberg, S. & Molin, S. (2002). Is there a role for quorum sensing signals in bacterial biofilms? *Current Opinion in Microbiology* **5**, 254-258.

Kojic, E. M. & Darouiche, R. O. (2004). *Candida* infections of medical devices. *Clinical Microbiology Reviews* **17**, 255-267.

Kolenbrander, P. E. (1997). Biofilm developmental biology. *Trends in Microbiology* **5**, 475-475.

Kontoyiannis, D. P., Vaziri, I., Hanna, H. A., Boktour, M., Thornby, J., Hachem, R., Bodey, G. P. & Raad, I. I. (2001). Risk factors for *Candida tropicalis* fungemia in patients with cancer. *Clinical Infectious Diseases* **33**, 1676-1681.

Koo, H., Pearson, S. K., Scott-Anne, K., Abranches, J., Cury, J. A., Rosalen, P. L., Park, Y. K., Marquis, R. E. & Bowen, W. H. (2002). Effects of apigenin and tt-farnesol on glucosyltransferase activity, biofilm viability and caries development in rats. *Oral Microbiology and Immunology* **17**, 337-343.

Krishnamurthy, S., Gupta, V., Prasad, R., Panwar, S. L. & Prasad, R. (1998). Expression of *CDR1*, a multidrug resistance gene of *Candida albicans*: Transcriptional activation by heat shock, drugs and human steroid hormones. *FEMS Microbiology Letters* **160**, 191-197.

Kruppa, M., Krom, B. P., Chauhan, N., Bambach, A. V., Cihlar, R. L. & Calderone, R. A. (2004). The two-component signal transduction protein *Chk1p* regulates quorum sensing in *Candida albicans*. *Eukaryotic Cell* **3**, 1062-1065.

Kruppa, M., Goins, T., Cutler, J. E., Lowman, D., Williams, D., Chauhan, N., Menon, V., Singh, P., Li, D. M. & Calderone, R. (2003). The role of the *Candida albicans* histidine kinase (*CHK1*) gene in the regulation of cell wall mannan and glucan biosynthesis. *FEMS Yeast Research* **4**, 125-125.

Kuhn, D. M., Chandra, J., Mukherjee, P. K. & Ghannoum, M. A. (2002a). Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. *Infection and Immunity* **70**, 878-888.

Kuhn, D. M., George, T., Chandra, J., Mukherjee, P. K. & Ghannoum, M. A. (2002b). Antifungal susceptibility of *Candida* biofilms: Unique efficacy of amphotericin B lipid formulations and echinocandins. *Antimicrobial Agents and Chemotherapy* **46**, 1773-1780.

Kumamoto, C. A. (2005). A contact-activated kinase signals *Candida albicans* invasive growth and biofilm development. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 5576-5581.

Kumamoto, C. A. & Vinces, M. D. (2005). Contributions of hyphae and hypha-co-regulated genes to *Candida albicans* virulence. *Cellular Microbiology* **7**, 1546-1554.

Kupferwasser, L. I., Yeaman, M. R., Shapiro, S. M., Nast, C. C., Sullam, P. M., Filler, S. G. & Bayer, A. S. (1999). Acetylsalicylic acid reduces vegetation bacterial density, hematogenous bacterial dissemination, and frequency of embolic events in experimental *Staphylococcus aureus* endocarditis through antiplatelet and antibacterial effects. *Circulation* **99**, 2791-2797.

Kupferwasser, L. I., Yeaman, M. R., Nast, C. C., Kupferwasser, D., Xiong, Y. Q., Palma, M., Cheung, A. L. & Bayer, A. S. (2003). Salicylic acid attenuates virulence in endovascular infections by targeting global regulatory pathways in *Staphylococcus aureus*. *Journal of Clinical Investigation* **112**, 222-233.

Laffey, S. F. & Butler, G. (2005). Phenotype switching affects biofilm formation by *Candida parapsilosis*. *Microbiology* **151**, 1073-1081.

Latge, J. P. & Calderone, R. (2002). Host-microbe interactions: fungi invasive human fungal opportunistic infections - Editorial overview. *Current Opinion in Microbiology* **5**, 355-358.

Leidich, S. D., Ibrahim, A. S., Fu, Y., Koul, A., Jessup, C., Vitullo, J., Fonzi, W., Mirbod, F., Nakashima, S., Nozawa, Y. & Ghannoum, M. A. (1998). Cloning and disruption of caPLB1, a phospholipase B gene involved in the pathogenicity of *Candida albicans*. *Journal of Biological Chemistry* **273**, 26078-26086.

Lengeler, K. B., Davidson, R. C., D'Souza, C., Harashima, T., Shen, W. C., Wang, P., Pan, X. W., Waugh, M. & Heitman, J. (2000). Signal transduction cascades regulating fungal development and virulence. *Microbiology and Molecular Biology Reviews* **64**, 746-785.

Lewis, K. (2001). Riddle of biofilm resistance. *Antimicrobial Agents and Chemotherapy* **45**, 999-1007.

Lewis, K. (2005). Persister cells and the riddle of biofilm survival. *Biochemistry-Moscow* **70**, 267-274.

Lewis, R. E., Lo, H. J., Raad, I. I. & Kontoyiannis, D. P. (2002). Lack of catheter infection by the *efg1/efg1 cph1/cph1* double-null mutant, a *Candida albicans* strain that is defective in filamentous growth. *Antimicrobial Agents and Chemotherapy* **46**, 1153-1155.

Liu, H. P. (2001). Transcriptional control of dimorphism in *Candida albicans*. *Current Opinion in Microbiology* **4**, 728-735.

Liu, H. P., Kohler, J. & Fink, G. R. (1994). Suppression of hyphal formation in *Candida albicans* by mutation of a Ste12 homolog. *Science* **266**, 1723-1726.

Lo, H. J., Kohler, J. R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A. & Fink, G. R. (1997). Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90**, 939-949.

Loose, D. S., Schurman, D. J. & Feldman, D. (1981). A corticosteroid binding-protein and endogenous ligand in *C. albicans* indicating a possible steroid-receptor system. *Nature* **293**, 477-479.

Lyon, G. J. & Novick, R. P. (2004). Peptide signaling in *Staphylococcus aureus* and other gram-positive bacteria. *Peptides* **25**, 1389-1403.

Machida, K., Tanaka, T., Yano, Y., Otani, S. & Taniguchi, M. (1999). Farnesol-induced growth inhibition in *Saccharomyces cerevisiae* by a cell cycle mechanism. *Microbiology* **145**, 293-299.

Mah, T. F. C. & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends in Microbiology* **9**, 34-39.

Marr, K. A., Rustad, T. R., Rex, J. H. & White, T. C. (1999). The trailing end point phenotype in antifungal susceptibility testing is pH dependent. *Antimicrobial Agents and Chemotherapy* **43**, 1383-1386.

Martin, S. W., Douglas, L. M. & Konopka, J. B. (2005). Cell cycle dynamics and quorum sensing in *Candida albicans* chlamydospores are distinct from budding and hyphal growth. *Eukaryotic Cell* **4**, 1191-1202.

Masako, K., Hideyuki, I., Shigeyuki, O. & Zenro, I. (2005a). A novel method to control the balance of skin microflora - Part 1. Attack on biofilm of *Staphylococcus aureus* without antibiotics. *Journal of Dermatological Science* **38**, 197-205.

Masako, K., Yusuke, K., Hideyuki, I., Atsuko, M., Yoshiki, M., Kayoko, M. & Makoto, K. (2005b). A novel method to control the balance of skin microflora - Part 2. A study to assess the effect of a cream containing farnesol and xylitol on atopic dry skin. *Journal of Dermatological Science* **38**, 207-213.

Masuoka, J. (2004). Surface glycans of *Candida albicans* and other pathogenic fungi: Physiological roles, clinical uses, and experimental challenges. *Clinical Microbiology Reviews* **17**, 281-310.

McCourtie, J. & Douglas, L. J. (1984). Relationship between cell-surface composition, adherence, and virulence of *Candida albicans*. *Infection and Immunity* **45**, 6-12.

McGeady, P., Logan, D. A. & Wansley, D. L. (2002). A protein-farnesyl transferase inhibitor interferes with the serum-induced conversion of *Candida albicans* from a cellular yeast form to a filamentous form. *FEMS Microbiology Letters* **213**, 41-44.

McLain, N. & Dolan, J. W. (1997). Phospholipase D activity is required for dimorphic transition in *Candida albicans*. *Microbiology* **143**, 3521-3526.

McLean, R. J. C., Whiteley, M., Stickler, D. J. & Fuqua, W. C. (1997). Evidence of autoinducer activity in naturally occurring biofilms. *FEMS Microbiology Letters* **154**, 259-263.

Miller, M. B. & Bassler, B. L. (2001). Quorum sensing in bacteria. *Annual Review of Microbiology* **55**, 165-199.

Mitchell, A. P. (1998). Dimorphism and virulence in *Candida albicans*. *Current Opinion in Microbiology* **1**, 687-692.

Moller, S., Pedersen, A. R., Poulsen, L. K., Arvin, E. & Molin, S. (1996). Activity and three-dimensional distribution of toluene-degrading *Pseudomonas putida* in a multispecies biofilm assessed by quantitative in situ hybridization and scanning confocal laser microscopy. *Applied and Environmental Microbiology* **62**, 4632-4640.

Moosa, M. Y. S., Sobel, J. D., Elhalis, H., Du, W. J. & Akins, R. A. (2004). Fungicidal activity of fluconazole against *Candida albicans* in a synthetic vagina-simulative medium. *Antimicrobial Agents and Chemotherapy* **48**, 161-167.

Moran, G. P., Sullivan, D. J., Henman, M. C., McCreary, C. E., Harrington, B. J., Shanley, D. B. & Coleman, D. C. (1997). Antifungal drug susceptibilities of oral *Candida dubliniensis* isolates from human immunodeficiency virus (HIV)-infected and non-HIV-infected subjects and generation of stable fluconazole-resistant derivatives *in vitro*. *Antimicrobial Agents and Chemotherapy* **41**, 617-623.

Mosel, D. D., Dumitru, R., Hornby, J. M., Atkin, A. L. & Nickerson, K. W. (2005). Farnesol concentrations required to block germ tube formation in *Candida albicans* in the presence and absence of serum. *Applied and Environmental Microbiology* **71**, 4938-4940.

Mukherjee, P. K., Chandra, J., Kuhn, D. A. & Ghannoum, M. A. (2003). Mechanism of fluconazole resistance in *Candida albicans* biofilms: Phase-specific role of efflux pumps and membrane sterols. *Infection and Immunity* **71**, 4333-4340.

Muller, E., Al-Attar, J., Wolff, A. G. & Farber, B. F. (1998). Mechanism of salicylate-mediated inhibition of biofilm in *Staphylococcus epidermidis*. *Journal of Infectious Diseases* **177**, 501-503.

Munro, C. A., Bates, S., Buurman, E. T., Hughes, H. B., MacCallum, D. M., Bertram, G., Atrih, A., Ferguson, M. A. J., Bain, J. M., Brand, A., Hamilton, S., Westwater, C., Thomson, L. M., Brown, A. J. P., Odds, F. C. & Gow, N. A. R. (2005). *Mnt1p* and *Mnt2p* of *Candida albicans* are partially redundant alpha-1,2-mannosyltransferases that participate in O-linked mannosylation and are required for adhesion and virulence. *Journal of Biological Chemistry* **280**, 1051-1060.

Naglik, J. R., Rodgers, C. A., Shirlaw, P. J., Dobbie, J. L., Fernandes-Naglik, L. I., Greenspan, D., Agabian, N. & Challacombe, S. J. (2003). Differential expression of *Candida albicans* secreted aspartyl proteinase and phospholipase B genes in humans correlates with active oral and vaginal infections. *Journal of Infectious Diseases* **188**, 469-479.

Narayanan, T. K. & Rao, G. R. (1976). Production of beta-(4-hydroxyphenyl) ethanol and beta-(4-hydroxyphenyl) lactic acid by *Candida* Species. *Canadian Journal of Microbiology* **22**, 384-389.

NCCLS (1995). Reference method for broth dilution antifungal susceptibility testing of yeasts; Tentative standard. *National Committee for Clinical Laboratory Standards, Villanova, Pa.*

Nealson, K. H. & Hastings, J. W. (1979). Bacterial bioluminescence - Its control and ecological significance. *Microbiological Reviews* **43**, 496-518.

Neugnot, V., Moulin, G., Dubreucq, E. & Bigey, F. (2002). The lipase/acyltransferase from *Candida parapsilosis* - Molecular cloning and characterization of purified recombinant enzymes. *European Journal of Biochemistry* **269**, 1734-1745.

Nicolau, D. P., Marangos, M. N., Nightingale, C. H. & Quintiliani, R. (1995). Influence of aspirin on development and treatment of experimental

Staphylococcus aureus endocarditis. *Antimicrobial Agents and Chemotherapy* **39**, 1748-1751.

Nicolle, L. E. (2005). Catheter-related urinary tract infection. *Drugs and Aging* **22**, 627-639.

Niimi, K., Niimi, M., Shepherd, M. G. & Cannon, R. D. (1997). Regulation of N-acetylglucosaminidase production in *Candida albicans*. *Archives of Microbiology* **168**, 464-472.

Nikawa, H., Nishimura, H., Yamamoto, T., Hamada, T. & Samaranayake, L. P. (1996). The role of saliva and serum in *Candida albicans* biofilm formation on denture acrylic surfaces. *Microbial Ecology in Health and Disease* **9**, 35-48.

Nilsson, P., Olofsson, A., Fagerlind, M., Fagerstrom, T., Rice, S., Kjelleberg, S. & Steinberg, P. (2001). Kinetics of the AHL regulatory system in a model biofilm system: How many bacteria constitute a "quorum"? *Journal of Molecular Biology* **309**, 631-640.

Nivoix, Y., Zamfir, A., Lutun, P., Kara, F., Remy, V., Lioure, B., Rigolot, J. C., Entz-Werle, N., Letscher-Bru, V., Waller, J., Leveque, D., Koffel, J. C., Beretz, L. & Herbrecht, R. (2006). Combination of caspofungin and an azole or an amphotericin B formulation in invasive fungal infections. *Journal of Infection* **52**, 67-74.

Nohmi, T., Abe, S., Tansho, S. & Yamaguchi, H. (1994). Suppression of anticandida activity of murine and human neutrophils by glucocorticoids. *Microbiology and Immunology* **38**, 977-982.

Nohmi, T., Abe, S., Dobashi, K., Tansho, S. & Yamaguchi, H. (1995). Suppression of anticandida activity of murine neutrophils by progesterone *in vitro*

- a possible mechanism in pregnant womens vulnerability to vaginal candidiasis. *Microbiology and Immunology* **39**, 405-409.

Noverr, M. C., Toews, G. B. & Huffnagle, G. B. (2002). Production of prostaglandins and leukotrienes by pathogenic fungi. *Infection and Immunity* **70**, 400-402.

Noverr, M. C., Erb-Downward, J. R. & Huffnagle, G. B. (2003). Production of eicosanoids and other oxylipins by pathogenic eukaryotic microbes. *Clinical Microbiology Reviews* **16**, 517-533.

Noverr, M. C., Phare, S. M., Toews, G. B., Coffey, M. J. & Huffnagle, G. B. (2001). Pathogenic yeasts *Cryptococcus neoformans* and *Candida albicans* produce immunomodulatory prostaglandins. *Infection and Immunity* **69**, 2957-2963.

Odds, F. (1988). *Candida and candidosis*. London: Bailliere Tindall.

Oh, K. B., Miyazawa, H., Naito, T. & Matsuoka, H. (2001). Purification and characterization of an autoregulatory substance capable of regulating the morphological transition in *Candida albicans*. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 4664-4668.

Okabe, S., Satoh, H. & Watanabe, Y. (2001). Analysis of microbial structure and function of nitrifying biofilms. *Methods in enzymology* **337**, 213-224.

Owen, D. H. & Katz, D. F. (1999). A vaginal fluid simulant. *Contraception* **59**, 91-95.

Pendrak, M. L. & Klotz, S. A. (1995). Adherence of *Candida albicans* to Host-Cells. *FEMS Microbiology Letters* **129**, 103-113.

Pfaller, M. A., Jones, R. N., Doern, G. V., Sader, H. S., Hollis, R. J., Messer, S. A. & Grp, S. P. (1998). International surveillance of bloodstream infections due to *Candida* species: Frequency of occurrence and antifungal susceptibilities of isolates collected in 1997 in the United States, Canada, and South America for the SENTRY program. *Journal of Clinical Microbiology* **36**, 1886-1889.

Pfaller, M. A., Diekema, D. J., Jones, R. N., Sader, H. S., Fluit, A. C., Hollis, R. J., Messer, S. A. & Grp, S. P. (2001). International surveillance of bloodstream infections due to *Candida species*: Frequency of occurrence and *in vitro* susceptibilities to fluconazole, ravuconazole, and voriconazole of isolates collected from 1997 through 1999 in the SENTRY antimicrobial surveillance program. *Journal of Clinical Microbiology* **39**, 3254-3259.

Pina-Vaz, C., Rodrigues, A. G., Costa-de-Oliveira, S., Ricardo, E. & Mardh, P. A. (2005). Potent synergic effect between ibuprofen and azoles on *Candida* resulting from blockade of efflux pumps as determined by FUN-1 staining and flow cytometry. *Journal of Antimicrobial Chemotherapy* **56**, 678-685.

Pina-Vaz, C., Sansonetty, F., Rodrigues, A. G., Martinez-De-Oliveira, J., Fonseca, A. F. & Mardh, P. A. (2000). Antifungal activity of ibuprofen alone and in combination with fluconazole against *Candida* species. *Journal of Medical Microbiology* **49**, 831-840.

Pizzo, G., Giuliana, G., Milici, M. E. & Giangreco, R. (2000). Effect of dietary carbohydrates on the *in vitro* epithelial adhesion of *Candida albicans*, *Candida tropicalis*, and *Candida krusei*. *Microbiologica* **23**, 63-71.

Price, C. T. D., Lee, I. R. & Gustafson, J. E. (2000). The effects of salicylate on bacteria. *International Journal of Biochemistry and Cell Biology* **32**, 1029-1043.

Ramage, G., Vande Walle, K., Wickes, B. L. & Lopez-Ribot, J. L. (2001a). Biofilm formation by *Candida dubliniensis*. *Journal of Clinical Microbiology* **39**, 3234-3240.

Ramage, G., vande Walle, K., Wickes, B. L. & Lopez-Ribot, J. L. (2001b). Standardized method for *in vitro* antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrobial Agents and Chemotherapy* **45**, 2475-2479.

Ramage, G., Vandewalle, K., Wickes, B. L. & Lopez-Ribot, J. L. (2001c). Characteristics of biofilm formation by *Candida albicans*. *Revista Iberoamericana De Micologia: Organo De La Asociacion Espanola De Especialistas En Micologia* **18**, 163-170.

Ramage, G., Saville, S. P., Wickes, B. L. & Lopez-Ribot, J. L. (2002a). Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum-sensing molecule. *Applied and Environmental Microbiology* **68**, 5459-5463.

Ramage, G., VandeWalle, K., Lopez-Ribot, J. L. & Wickes, B. L. (2002b). The filamentation pathway controlled by the *Efg1* regulator protein is required for normal biofilm formation and development in *Candida albicans*. *FEMS Microbiology Letters* **214**, 95-100.

Ramage, G., Bachmann, S., Patterson, T. F., Wickes, B. L. & Lopez-Ribot, J. L. (2002c). Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. *Journal of Antimicrobial Chemotherapy* **49**, 973-980.

Ramon, A. M., Porta, A. & Fonzi, W. A. (1999). Effect of environmental pH on morphological development of *Candida albicans* is mediated via the PacC-related transcription factor encoded by PRR2. *Journal of Bacteriology* **181**, 7524-7530.

Redding, S. W. (2001). The role of yeasts other than *Candida albicans* in oropharyngeal candidiasis. *Current Opinion in Infectious Diseases* **14**, 673-677.

Rex, J. H., Rinaldi, M. G. & Pfaller, M. A. (1995). Resistance of *Candida* species to fluconazole. *Antimicrobial Agents and Chemotherapy* **39**, 1-8.

Rex, J. H., Pfaller, M. A., Rinaldi, M. G., Polak, A. & Galgiani, J. N. (1993). Antifungal susceptibility testing. *Clinical Microbiology Reviews* **6**, 367-381.

Rex, J. H., Nelson, P. W., Paetznick, V. L., Lozano-Chiu, M., Espinel-Ingroff, A. & Anaissie, E. J. (1998). Optimizing the correlation between results of testing *in vitro* and therapeutic outcome *in vivo* for fluconazole by testing critical isolates in a murine model of invasive candidiasis. *Antimicrobial Agents and Chemotherapy* **42**, 129-134.

Richard, M. L., Nobile, C. J., Bruno, V. M. & Mitchell, A. P. (2005). *Candida albicans* biofilm-defective mutants. *Eukaryotic Cell* **4**, 1493-1502.

Riggle, F. J., Andrutis, K. A., Chen, X., Tzipori, S. R. & Kumamoto, C. A. (1999). Invasive lesions containing filamentous forms produced by a *Candida albicans* mutant that is defective in filamentous growth in culture. *Infection and Immunity* **67**, 3649-3652.

Rioboo-Crespo Mdel, R., Planells-del Pozo, P. & Rioboo-Garcia, R. (2005). Epidemiology of the most common oral mucosal diseases in children. *Oral Medicine and Pathology* **10**, 376-387.

Robert, R., Nail, S., Marot-Leblond, A., Cottin, J., Miegeville, M., Quenouillere, S., Mahaza, C. & Senet, J. M. (2000). Adherence of platelets to *Candida* species *in vivo*. *Infection and Immunity* **68**, 570-576.

Roberts, A., Matthews, J. B., Socransky, S. S., Freestone, P. P. E., Williams, P. H. & Chapple, I. L. C. (2002). Stress and the periodontal diseases: effects of catecholamines on the growth of periodontal bacteria *in vitro*. *Oral Microbiology and Immunology* **17**, 296-303.

Roberts, A., Matthews, J. B., Socransky, S. S., Freestone, P. P. E., Williams, P. H. & Chapple, I. L. C. (2005). Stress and the periodontal diseases: growth responses of periodontal bacteria to *Escherichia coli* stress-associated autoinducer and exogenous Fe. *Oral Microbiology and Immunology* **20**, 147-153.

Roberts, M. E. & Stewart, P. S. (2005). Modelling protection from antimicrobial agents in biofilms through the formation of persister cells. *Microbiology* **151**, 75-80.

Rosner, J. L. (1985). Nonheritable resistance to chloramphenicol and other antibiotics induced by salicylates and other chemotactic repellents in *Escherichia coli* K-12. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 8771-8774.

Roustan, J. L., Chu, A. R., Moulin, G. & Bigey, F. (2005). A novel lipase/acyltransferase from the yeast *Candida albicans*: expression and characterisation of the recombinant enzyme. *Applied Microbiology and Biotechnology* **68**, 203-212.

Samaranayake, Y. H., Ye, J., Yau, J. Y. Y., Cheung, B. P. K. & Samaranayake, L. P. (2005). *In vitro* method to study antifungal perfusion in *Candida* biofilms. *Journal of Clinical Microbiology* **43**, 818-825.

Sampedro, I., Romero, C., Ocampo, J. A., Brenes, M. & Garcia, I. (2004). Removal of monomeric phenols in dry mill olive residue by saprobic fungi. *Journal of Agricultural and Food Chemistry* **52**, 4487-4492.

Sanchez-Martinez, C. & Perez-Martin, J. (2001). Dimorphism in fungal pathogens: *Candida albicans* and *Ustilago maydis* - similar inputs, different outputs. *Current Opinion in Microbiology* **4**, 214-221.

Sangeorzan, J. A., Bradley, S. F., He, X. G., Zarins, L. T., Ridenour, G. L., Tiballi, R. N. & Kauffman, C. A. (1994). Epidemiology of oral candidiasis in HIV infected patients: Colonization, infection, treatment, and emergence of fluconazole resistance. *American Journal of Medicine* **97**, 339-346.

Schaller, M., Borelli, C., Korting, H. C. & Hube, B. (2005). Hydrolytic enzymes as virulence factors of *Candida albicans*. *Mycoses* **48**, 365-377.

Schierholz, J. M., Beuth, J. & Pulverer, G. (1999a). Silver coating of medical devices for catheter-associated infections? *American Journal of Medicine* **107**, 101-102.

Schierholz, J. M., Seyfert, U. T., Rump, A. F. E., Beuth, J. & Pulverer, G. (1999b). Strategies for the prevention of catheter material-associated thrombosis and bloodstream infections. *Infusionstherapie Und Transfusionsmedizin* **26**, 278-287.

Schinabeck, M. K., Long, L. A., Hossain, M. A., Chandra, J., Mukherjee, P. K., Mohamed, S. & Ghannoum, M. A. (2004). Rabbit model of *Candida albicans* biofilm infection: Liposomal amphotericin B antifungal lock therapy. *Antimicrobial Agents and Chemotherapy* **48**, 1727-1732.

Schmitt, H. J. (1993). New methods of delivery of amphotericin-B. *Clinical Infectious Diseases* **17**, S501-S506.

Schofield, D. A., Westwater, C., Warner, T. & Balish, E. (2005). Differential *Candida albicans* lipase gene expression during alimentary tract colonization and infection. *FEMS Microbiology Letters* **244**, 359-365.

Schreiber, W., Olbrisch, A., Vorwerk, C. K., Konig, W. & Behrens-Baumann, W. (2003). Combined topical fluconazole and corticosteroid treatment for experimental *Candida albicans* keratomycosis. *Investigative Ophthalmology and Visual Science* **44**, 2634-2643.

Scott, E. M., Tariq, V. N. & Mccrory, R. M. (1995). Demonstration of synergy with fluconazole and either ibuprofen, sodium-salicylate, or propylparaben against *Candida albicans in vitro*. *Antimicrobial Agents and Chemotherapy* **39**, 2610-2614.

Sentheshanmuganathan, S. & Elsden, S. R. (1958). Mechanism of the formation of tyrosol by *saccharomyces cerevisiae*. *Biochemical Journal* **69**, 210-218.

Shchepin, R., Dumitru, R., Nickerson, K. W., Lund, M. & Dussault, P. H. (2005). Biologically active fluorescent farnesol analogs. *Chemistry and Biology* **12**, 639-641.

Shchepin, R., Hornby, J. M., Burger, E., Niessen, T., Dussault, P. & Nickerson, K. W. (2003). Quorum sensing in *Candida albicans*: Probing farnesol's mode of action with 40 natural and synthetic farnesol analogs. *Chemistry and Biology* **10**, 743-750.

Singh, N. (2001). Trends in the epidemiology of opportunistic fungal infections: Predisposing factors and the impact of antimicrobial use practices. *Clinical Infectious Diseases* **33**, 1692-1696.

Skerl, K. G., Calderone, R. A., Segal, E., Sreevalsan, T. & Scheld, W. M. (1984). Invitro binding of *Candida albicans* yeast cells to human fibronectin. *Canadian Journal of Microbiology* **30**, 221-227.

Slavin, M. A., Szer, J., Grigg, A. P., Roberts, A. W., Seymour, J. F., Sasadeusz, J., Thursky, K., Chen, S. C., Morrissey, C. O., Heath, C. H. & Sorrell, T. (2004). Guidelines for the use of antifungal agents in the treatment of invasive *Candida* and mould infections. *Internal Medicine Journal* **34**, 301-301.

Slininger, P. J., Burkhead, K. D. & Schisler, D. A. (2004). Antifungal and sprout regulatory bioactivities of phenylacetic acid, indole-3-acetic acid, and tyrosol isolated from the potato dry rot suppressive bacterium *Enterobacter cloacae* S11 : T : 07. *Journal of Industrial Microbiology and Biotechnology* **31**, 517-524.

Sobel, J. D. (1998). Vulvovaginitis due to *Candida glabrata*. An emerging problem. *Mycoses* **41**, 18-22.

Song, J. W., Shin, J. H., Shin, D. H., Jung, S. I., Cho, D., Kee, S. J., Shin, M. G., Suh, S. P. & Ryang, D. W. (2005). Differences in biofilm production by three genotypes of *Candida parapsilosis* from clinical sources. *Medical Mycology* **43**, 657-661.

Stehr, F., Kretschmar, M., Kroger, C., Hube, B. & Schafer, W. (2003). Microbial lipases as virulence factors. *Journal of Molecular Catalysis B-Enzymatic* **22**, 347-355.

Stern, G. A. & Buttross, M. (1991). Use of corticosteroids in combination with antimicrobial drugs in the treatment of infectious corneal disease. *Ophthalmology* **98**, 847-853.

Stewart, P. S. (1996). Theoretical aspects of antibiotic diffusion into microbial biofilms. *Antimicrobial Agents and Chemotherapy* **40**, 2517-2522.

Stickler, D. J., Morris, N. S., McLean, R. J. C. & Fuqua, C. (1998). Biofilms on indwelling urethral catheters produce quorum-sensing signal molecules in situ and *in vitro*. *Applied and Environmental Microbiology* **64**, 3486-3490.

Stoldt, V. R., Sonneborn, A., Leuker, C. E. & Ernst, J. F. (1997). *Efg1p*, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. *EMBO Journal* **16**, 1982-1991.

Stover, E. P., Loose, D. S., Stevens, D. A. & Feldman, D. (1983). Ketoconazole binds to the intracellular corticosteroid-binding protein in *Candida albicans*. *Biochemical and Biophysical Research Communications* **117**, 43-50.

Tatman, D. & Mo, H. B. (2002). Volatile isoprenoid constituents of fruits, vegetables and herbs cumulatively suppress the proliferation of murine B16 melanoma and human HL-60 leukemia cells. *Cancer Letters* **175**, 129-139.

Taylor, M. M., MacDonald, K., Morris, A. J. & McMaster, C. R. (2005). Enhanced apoptosis through farnesol inhibition of phospholipase D signal transduction. *FEBS Journal* **272**, 5056-5063.

Te Dorsthorst, D. T. A., Verweij, P. E., Meis, J. F. G. M. & Mouton, J. W. (2005). Relationship between *in vitro* activities of amphotericin B and flucytosine and pH for clinical yeast and mold isolates. *Antimicrobial Agents and Chemotherapy* **49**, 3341-3346.

Te Dorsthorst, D. T. A., Mouton, J. W., van den Beukel, C. J. P., van der Lee, H. A. L., Meis, J. F. G. M. & Verweij, P. E. (2004). Effect of pH on the *in vitro* activities of amphotericin B, itraconazole, and flucytosine against *Aspergillus isolates*. *Antimicrobial Agents and Chemotherapy* **48**, 3147-3150.

Theaker, E. D., Drucker, D. B. & Gibbs, A. C. C. (1993). The possible influence of the menstrual-cycle on the adherence of *Candida albicans* to human buccal epithelial-cells *in vitro*. *Archives of Oral Biology* **38**, 353-355.

Thinkhamrop, J., Lumbiganon, P., Thongkrajai, P., Chongsomchai, C. & Pakarasang, M. (1999). Vaginal fluid pH as a screening test for vaginitis. *International Journal of Gynecology and Obstetrics* **66**, 143-148.

van Burik, J. A. H. & Magee, P. T. (2001). Aspects of fungal pathogenesis in humans. *Annual Review of Microbiology* **55**, 743-772.

Vardar-Unlu, G., McSharry, C. & Douglas, L. J. (1998). Fucose-specific adhesins on germ tubes of *Candida albicans*. *FEMS Immunology and Medical Microbiology* **20**, 55-67.

Wagner, T., Borgvonzepelin, M. & Ruchel, R. (1995). pH-dependent denaturation of extracellular aspartic proteinases from *Candida* species. *Journal of Medical and Veterinary Mycology* **33**, 275-278.

Wang, W. H., Wong, W. M., Dailidiene, D., Berg, D. E., Gu, Q., Lai, K. C., Lam, S. K. & Wong, B. C. Y. (2003). Aspirin inhibits the growth of *Helicobacter pylori* and enhances its susceptibility to antimicrobial agents. *Gut* **52**, 490-495.

Warnock, D. W. (1992). Azole drug-resistance in *Candida* species. *Journal of Medical Microbiology* **37**, 225-226.

Webb, B. C., Thomas, C. J., Willcox, M. D. P., Harty, D. W. S. & Knox, K. W. (1998). *Candida*-associated denture stomatitis. Aetiology and management: A review. Part 1. Factors influencing distribution of candida species in the oral cavity. *Australian Dental Journal* **43**, 45-50.

Wegner, B., Baer, P., Gauer, S., Oremek, G., Hauser, I. A. & Geiger, H. (2005). Caspofungin is less nephrotoxic than amphotericin B *in vitro* and predominantly damages distal renal tubular cells. *Nephrology Dialysis Transplantation* **20**, 2071-2079.

Westwater, C., Balish, E. & Schofield, D. A. (2005). *Candida albicans* conditioned medium protects yeast cells from oxidative stress: A possible link between quorum sensing and oxidative stress resistance. *Eukaryot Cell* **4**, 1654-1661.

White, D. J., Habib, A. R., Vanthuyne, A., Langford, S. & Symonds, M. (2001). Combined topical flucytosine and amphotericin B for refractory vaginal Candida glabrata infections. *Sexually Transmitted Infections* **77**, 212-213.

White, S. & Larsen, B. (1997). *Candida albicans* morphogenesis is influenced by estrogen. *Cellular and Molecular Life Sciences* **53**, 744-749.

Wingard, J. R. & Leather, H. (2004). A new era of antifungal therapy. *Biology of Blood and Marrow Transplantation* **10**, 73-90.

Worthington, T. & Elliott, T. S. J. (2005). Diagnosis of central venous catheter related infection in adult patients. *Journal of Infection* **51**, 267-280.

Wu, K. K. (2000). Aspirin and salicylate - An old remedy with a new twist. *Circulation* **102**, 2022-2023.

Xu, J. F., Su, Z. G. & Feng, P. S. (1998). Production of salidroside through biotransformation of exogenous tyrosol in *Rhodiola sachalinensis* cell suspension cultures. *ACTA Botanica Sinica* **40**, 1129-1135.

Xu, X. M., Sansores-Garcia, L., Chen, X. M., Matijevic-Aleksic, N., Du, M. & Wu, K. K. (1999). Suppression of inducible cyclooxygenase 2 gene transcription

by aspirin and sodium salicylate. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 5292-5297.

Yamada-Okabe, T., Mio, T., Ono, N., Kashima, Y., Matsui, M., Arisawa, M. & Yamada-Okabe, H. (1999). Roles of three histidine kinase genes in hyphal development and virulence of the pathogenic fungus *Candida albicans*. *Journal of Bacteriology* **181**, 7243-7247.

Yucesoy, M., Oktem, I. M. A. & Gulay, Z. (2000). In-vitro synergistic effect of fluconazole with nonsteroidal anti-inflammatory agents against *Candida albicans* strains. *Journal of Chemotherapy* **12**, 385-389.

APPENDICES

Appendix 1. Composition of Growth Media

App. 1.1 Yeast Nitrogen Base (YNB; Difco)

Chemical	Wt/litre
Ammonium sulfate	5 g
Monopotassium phosphate	1 g
Magnesium sulfate	0.5 g
Sodium chloride	0.1 g
L-Histidine monohydrochloride	10 mg
LD Methionine	20 mg
LD-Trypotophan	20 mg
Inoslitol	2000 µg
Boric acid	500 µg
Niacin	400 µg
Manganese sulfate	400 µg
Pyridoxine HCL	400 µg
Zinc sulfate	400 µg
Thiamine HCL	400 µg
Calcium pantothenate	400 µg
Ferric chloride	200 µg
Sodium molybdate	200 µg
Riboflavin	200 µg
p-Aminobenzoic acid	200 µg
Potassium Iodide	100 µg
Copper sulfate	40 µg
Folic acid	2 µg
Biotin	2 µg

Glucose was added to a final concentration of 50 mM.
Final pH 5.4 ± 0.1.

App. 1.2 Sabouraud Dextrose Broth (SDB;Oxoid)

Chemical	g/litre
Neopeptone	10
Bacto dextrose	40

Final pH 5.6 ± 0.2.

App. 1.3 Sabouraud Dextrose Agar (SDA;Oxoid)

Chemical	g/litre
Neopeptone	10
Bacto dextrose	40
Agar	15

Final pH 5.6 ± 0.2.

App. 1.4 Vagina-simulative medium (VS)

Chemical	g/litre
NaCl	3.51
KOH	1.40
Ca $(OH)_2$	0.222
Bovine serum albumin	0.018
Lactic acid	2.00
Acetic acid	1.00
Glycerol	0.16
Urea	0.4
Glucose	5.0

The pH was adjusted to 4.2 using HCl.

App. 1.5 RPMI 1640 with HEPES (Sigma)

Chemical	g/litre
$Ca(NO_3)_2 \cdot 4H_2O$	0.1
$MgSO_4$ (anhyd)	0.04884
NaCl	6.0
Na_2HPO_4 (anhyd)	0.8
AMINO ACIDS	
L-Arginine (free base)	0.2
L-Asparagine (anhyd)	0.05
L-Aspartic acid	0.02

L-Cystine•2HCl	0.0652
L-Glutamic acid	0.02
L-Glutamine	0.3
Glycine	0.01
L-Histidine (free base)	0.015
Hydroxy-L-Proline	0.02
L-Isoleucine	0.05
L-Leucine	0.05
L-Lysine•HCl	0.04
L-Methionine	0.015
L-Phenylalanine	0.015
L-Proline	0.02
L-Serine	0.03
L-Threonine	0.02
L-Tryptophan	0.005
L-Tyrosine•2Na•2H_2O	0.02883
L-Valine	0.02
VITAMINS	
D-Biotin	0.0002
Choline chloride	0.003
Folic acid	0.001
myo-Inositol	0.035
Niacinamide	0.001
p-Amino benzoic acid	0.001
D-Pantothenic acid•½Ca	0.00025
Pyridoxine•HCl	0.001
Riboflavin	0.0002
Thiamine•HCl	0.001
Vitamin B_{12}	0.000005
OTHER	
D-Glucose	2.0
Glutathione (reduced)	0.001
HEPES	5.96
Phenol Red•Na	0.0053
$NaHCO_3$	2.0

Final pH 7.0

App. 1.6 RPMI 1640 with L-Glutamine (Cambrex)

Chemical	**mg/litre**
Inorganic salts	
$Ca(NO_3)_2$•4H_2O	100
KCl	400
$MgSO_4$•7H_2O	100

NaCl	6000
$NaHCO_3$	2000
$Na_2HPO_4 \cdot 7H_2O$	1512
Other Components	
Glucose	2000
Glutathione	1
Phenol red•Na	5
Amino acids	
L-Arginine	200
L-Asparagine•H_2O	50
L-Aspartic acid	20
L-Cystine	50
L-Glutamic acid	20
L-Glutamine	300
Glycine	10
L-Histidine	15
Hydroxy L-proline	20
L-Isoleucine	50
L-Leucine	50
L-Lysine•HCl	40
L-Methionine	15
L-Phenylalanine	15
L-Proline	20
L-Serine	30
L-Threonine	20
L-Tryptophan	5
L-Tyrosine	20
L-Valine	20
Vitamins	
P- Aminobenzoic acid	1
D-Biotin	0.2
D-Ca Pantothenate	0.25
Choline chloride	3
Folic acid	1
i-Inositol	35
Nicotinamide	1
Pyridoxine•HCl	1
Riboflavin	0.20
Thiamine•HCl	1
Vitamin B_{12}	0.01

Final pH 7.0

App. 1.7 Yeast peptone dextrose (YPD) medium

Chemical	**g/litre**
Yeast extract	10
Peptone	20
Dextrose	20

Final pH 6.0

App. 1.8 Hornby medium

Solution	**Volume**
0.05 M potassium phosphate buffer	0.5 ml
20% glucose in 30 mM $MgSO_4$	0.5 ml
0.3 M L-proline	0.17 ml
0.1M GlcNAc	0.13 ml
Distilled water	3.7 ml

Final pH 6.5

App. 1.9 Germ-tube inducers

The following inducers were prepared in 50 mM potassium phosphate buffer (pH 6.5)

(i) 10mM proline (Sigma)

(ii) 2.5mM N-acetylglucosamine (Sigma)

(iii) 10mM proline + 2.5mM N-acetylglucosamine

(iv) 5% foetal bovine serum

Appendix 2. buffers

App. 2.1 0.01 M phosphate buffered saline pH 7.2 ± 0.2 (Sigma)

One tablet contains:

10 mM phosphate buffer

2.7 mM potassium chloride

137 mM sodium chloride

One tablet was dissolved in 200 ml distilled water.

App. 2.2 0.1M citric acid – 0.2M disodium hydrogen phosphate buffer

Solution	**g/litre**
(i) 0.1M citric acid	21.01
(ii) 0.2M $Na_2 HPO_4$	28.4

Two solutions (i & ii) were mixed to give the appropriate pH:

	(i) 0.1M citric acid	(ii) 0.2M $Na_2 HPO_4$
For pH:	ml	ml
3	79.45	20.55
4	61.45	38.55
5	48.50	51.50
6	36.85	63.51
7	17.65	82.35

App. 2.3 Tris maleate – sodium hydroxide buffer (pH 8)

Solution	**g/litre**
(i) Tris-maleate	
0.02 M Tris	2.4
0.2 M Maleic acid	23.4
(i) 0.2 M NaOH	
NaOH	8.0

For pH 8, 25ml Tris-maleate and 34.5 ml 0.2M NaOH were mixed together and diluted with distilled water to 100 ml.

App. 2.4 1 M phosphate buffer

Chemical	g/litre
K_2HPO_4	133
KH_2PO_4	32.2

The chemicals were dissolved in ultra pure water, final pH 7.4

App. 2.5 (10x) EIA (electroimmunoassay) buffer

The following chemicals were dissolved in 100 ml of 1 M phosphate buffer solution:

Chemical	g/100 ml
Bovine serum albumin (BSA)	1.0
NaCl	23.4
Ethylenediamine tetraacetic acid (EDTA)	0.37
NaN_3	0.1

Final pH 7.4