Ana Claudia Santos de Azevedo Izidoro

FATORES DE VIRULÊNCIA DE CEPAS DE *Candida albicans* ISOLADAS DE FUMANTES E NÃO-FUMANTES

CURITIBA
2007

Ana Claudia Santos de Azevedo Izidoro, CD

FATORES DE VIRULÊNCIA DE CEPAS DE *Candida albicans* ISOLADAS DE FUMANTES E NÃO-FUMANTES

VIRULENCE FACTORS OF *Candida albicans* STRAINS ISOLATED FROM SMOKERS AND NON-SMOKERS

Dissertação apresentada ao Programa de Pós - Graduação em Odontologia da Pontifícia Universidade Católica do Paraná, como parte dos requisitos para obtenção do Título de Mestre em Odontologia, Área de Estomatologia

Orientador: Prof. Dr. Edvaldo Antonio Ribeiro Rosa

Co-orientadora: Dra. Maria Ângela Naval Machado

CURITIBA
2007

Dedicatória

Ao meu esposo Fábio, que sempre me incentivou, apoiou e ajudou nos momentos mais difíceis, inclusive na realização do trabalho laboratorial. E por todos os momentos felizes que tem me proporcionado nestes anos de convivência.

À minha querida filha Beatriz, por trazer alegria a minha vida e por compreender meus momentos de ausência.

Amo vocês!

Agradecimento especial

A Deus, pela constante presença em minha vida, por seu amor, pela força nos momentos de dificuldade, pela coragem nos momentos de insegurança, por iluminar meu caminho e me ajudar a seguir em frente.

A Santa Clara, por ser apoio e segurança sempre, por cuidar da minha vida e do meu destino.

Aos meus amados pais Hélio e Edna, meus irmãos Marcelo e Leonardo e minhas duas preciosas sobrinhas Marcelle e Jéssica, pelo amor, carinho, confiança e apoio que me deram para que eu concluísse mais esta etapa.

Aos meus sogros Osório e Eliete, por toda ajuda e apoio que me deram durante a realização deste curso.

Aos parentes e amigos que me deram apoio e carinho, me ajudando a superar as dificuldades.

A querida amiga Andressa Marafon Semprebom, pelo empenho e dedicação em me ajudar a desenvolver esta pesquisa, pela amizade, carinho e respeito.

Muito Obrigada!

Agradecimentos

Ao Reitor da Pontifícia Universidade Católica do Paraná, Professor Ivo Clemente Juliatto; ao Decano do Centro de Ciências Biológicas e da Saúde, Professor Alberto Accioly Veiga e ao Diretor do Curso de Odontologia, Professor Monir Tacla, pelo acolhimento nesta instituição de ensino superior.

Ao Diretor do Programa de Pós-Graduação em Odontologia, Professor Sérgio Vieira, pela dedicação em aprimorar cada vez mais o ensino nesta instituição.

Ao Prof. Dr. Fernando Henrique Westphalen pela coordenação do mestrado, área de concentração em Estomatologia.

A co-orientadora Profª. Drª. Maria Angela Naval Machado, pelo acompanhamento, confiança, pelo incentivo ao estudo, e amor à carreira acadêmica. Modelo de dedicação ao trabalho e profissionalismo. E por ter assumido minha orientação com tento carinho e empenho.

Ao orientador Prof. Dr. Edvaldo Antonio Ribeiro Rosa, pelos conhecimentos transmitidos, pelo empenho e pelo acompanhamento diário, sem os quais seria impossível a realização desta pesquisa.

A Rosimeire Takaki Rosa, que com sua valiosa colaboração e conhecimento científico tornou realidade esta pesquisa.

A querida professora e amiga Profª. Drª. Marina de Oliveira Ribas e, por todo carinho, amizade, paciência, dedicação, oportunidades, respeito e empenho durante todo o curso. Mais que mestra, exemplo de pessoa e amiga de todas as horas.

Ao Prof. Dr. Sérgio Ignácio, pelo imenso auxílio, pela dedicação, competência, paciência e pelos ensinamentos da bioestatística.

Aos professores do Programa de Mestrado em Odontologia, que com muita dedicação nos transmitiram muitos ensinamentos, entre estes a importância da constante renovação de conhecimentos. Prof. Dr. Wilson Denis Martins, Prof. Dr. Antônio Adilson Soares de Lima, Profª. Drª. Luciana Reis de Azevedo, Profª. Drª. Ana Maria Trindade Grégio, Prof. Dr. Julio Cezar Bisinelli, Profª. Drª. Beatriz Helena Sottile França, Profª. Drª. Paula Cristina Trevilatto, Prof. Dr. Samuel Jorge Moysés, Profª. Drª. Simone Tetu Moysés e Prof. Dr. Paulo Henrique Couto Souza.

Aos queridos professores Santo Gentil Foroni, e Prof. Wilson Kenji Shiroma, pela confiança depositada durante as cirurgias, pelos conhecimentos transmitidos e pela amizade conquistada.

A querida amiga e colega de trabalho Profª. Lúcia Fátima de Castro Ávila pela amizade, paciência e pelos ensinamentos transmitidos durante todo o curso.

A professora Maria Helena Souza pelo apoio e incentivo à pesquisa.

Aos queridos amigos de turma, que contribuíram para a realização deste curso, cada um do seu jeito, mas sempre com muito carinho e respeito. Carla Spagliari Baioni, Thaís de Almeida Lanzoni, Roberta Targa Stramandinolli, Elcy Pinto de Arruda, Fernando Luís Zanferrari, Therezinha Pastre, Patrícia Almeida e Silvana da Silva, muito obrigada pelo apoio e pela amizade.

A todos os amigos e amigas da Ortodontia e da Dentística, pela amizade e pelos momentos que passamos juntos.

À grande amiga Neide Borges, pela amizade, compreensão, dedicação e apoio.

A Fernanda Baboni, pela grande colaboração na coleta de material para esta pesquisa.

Aos meus amigos Ruy e Valéria Terra, por todo apoio e pela amizade incondicional de tantos anos.

A minha amiga Juliana Cherem Knapik, pela presença sincera e leal nos momentos que mais precisei.

Ao TC Sidney Sendtko, pela paciência e compreensão, e por ajudar a conciliar trabalho e estudo.

A todos os voluntários que colaboraram com a realização deste trabalho, pois sem eles nunca seria possível à realização de pesquisas clínicas.

A todos os funcionários da Clínica de Odontologia da PUCPR, especialmente à Shirley, Aline, Irene, Silvana, Betinha, Mari, Allan e Maria por todo o auxílio prestado e amizade conquistada.

A todas as pessoas que, de qualquer maneira, contribuíram para a conclusão deste curso.

Muito obrigada!

SUMÁRIO

1. LISTA DE ABREVIATURAS E SIGLAS

C.	- *Candida*
et al.	- E outros (abreviatura de "*et alli"*)
g	- Grama
spp.	- Espécies
$MgCl_2$	- Cloreto de magnésio
mL	- Mililitro
mm	- Milímetro
NaCl	- Cloreto de sódio
ºC	- Grau Celsius
pH	- Potencial hidrogeniônico
Pz	- Zona de precipitação
q.s.p	- Quantidade suficiente para atingir determinado volume
µg/mL	- Microgramas por mililitro
Sap	- Secretoras de aspartil-protease
Z	- Zimotipo

. RESUMO

O cigarro de tabaco é considerado fator predisponente para várias doenças, inclusive para a candidose bucal. Ele contem substâncias que podem ser degradadas por enzimas destes fungos, servindo como nutrientes. A produção de enzimas histolíticas pelas espécies de *Candida* é um fator importante no desenvolvimento de doença. As enzimas podem prover acesso aos nutrientes do organismo, facilitando penetração dos fungos nas células do hospedeiro. O objetivo deste estudo foi avaliar a carga fúngica bucal e a secreção de enzimas histolíticas das espécies de *Candida* isoladas de adultos saudáveis não-fumantes e fumantes . Cem adultos (42 não-fumantes e 58 fumantes) participaram do estudo. Os espécimes foram coletados do dorso da língua e da mucosa jugal e identificados utilizando-se o meio de cultura CHROMagar®Candida. A positividade do gênero *Candida* foi de 28,57% nos indivíduos não-fumantes e de 39,65% fumantes (P = 0,254). *C. albicans* foi a espécie mais prevalente nos dois grupos, sendo isolada em 78,57% nos não-fumantes e 72,41% nos fumantes. Outras espécies encontradas foram *C. parapsilosis, C. glabrata e C. tropicalis.* Dos indivíduos positivos foi selecionada aleatoriamente uma cepa de cada espécie, totalizando 43 cepas, que foram avaliadas quanto à produção das enzimas aspartil-protease (Sap), fosfolipase, condroitinase, esterase-lipase e hemolisina. Devido a pequena amostra de cepas não-*albicans* obtida, somente as cepas de *Candida albicans* foram analisadas estatisticamente. A atividade enzimática foi maior no grupo dos não-fumantes para todas as enzimas estudadas, com diferença estatística significativa na atividade da enzima fosfolipase (P = 0,013). Concluímos que o tabagismo não afetou significativamente a positividade de *Candida* spp. e nem a atividade enzimática das cepas de *C. albicans*.

Palavras chave: *Candida albicans;* fumantes; enzimas; fosfolipase.

3. ABSTRACT

The cigarette smoke contains substances that can be degraded by enzymes of *Candida* species, been used like nutrients and converted to carcinogen end products. The purpose of this study was evaluated the carriage of *Candida* species and analyse the extracellular proteolytic activities of *Candida* isolates from healthy adults non-smokers and smokers. One hundred adults (42 non-smokers and 58 smokers) were studied. The samples were collected from intra oral swabs and smears from dorsum of tongue, right and left jugal mucosa each. The identification of species of *Candida* was made using the culture medium CHROMagar®Candida. The carriage of the gender *Candida* was 28,57% in the non-smokers individuals and 39,65% in the smokers (P = 0,254). *Candida albicans* was the most prevalent one between the two groups, being isolated in 78.57% in non-smokers, and 72.41% in smokers. Other species found were *C. parapsilosis, C. glabrata*, and *C. tropicalis*. From the positive individuals was random one strain of *C. albicans*, comprised 32 strains that were evaluated regarding the proteolytic activity of the following enzymes: secreted aspartyl-protease (Sap), phospholipase, chondroitinase, esterase-lipase, and haemolysin. Regarding the hydrolytic enzymes, there was bigger enzymatic activity in the group of non-smokers, in all studied enzymes, with significant statistical difference between both groups in the activity of phospholipase, that was bigger in the strains of non-smokers (P = 0,013). The results obtained in this study have shown that the smoking do not alter significantly the carriage of *Candida* spp., neither the enzymatic activity of *C. albicans* strains, except the activity of phospholipase.

Keywords: *Candida albicans*, smokers, enzymes, phospholipase.

4. ARTIGO EM PORTUGUÊS

PÁGINA DE TÍTULO

FATORES DE VIRULÊNCIA DE CEPAS DE *Candida albicans* ISOLADAS DE FUMANTES E NÃO-FUMANTES

Ana Claudia Santos de Azevedo Izidoro, Edvaldo Antônio Ribeiro Rosa*, Maria Ângela Naval Machado

*Autor responsável pela correspondência

Endereço: Pontifícia Universidade Católica do Paraná

Laboratório de Estomatologia - CCBS

Rua Imaculada Conceição, nr 1155 – Prado Velho – Curitiba - Paraná.

CEP: 80 215 - 901

Telefone comercial: (041) 3271-1497

Fax: (041) 3271-1405

E-mail: edvaldo.rosa@pucpr.br

RESUMO

O cigarro de tabaco tem sido considerado como um fator predisponente para várias doenças, inclusive para a candidose bucal. Ele contem substâncias que podem ser degradadas por enzimas das espécies de *candida,* servindo como nutrientes. Por outro lado, a produção de enzimas histolíticas pelas espécies de *Candida* é um fator importante no desenvolvimento de doenças provocadas por fungos oportunistas. As enzimas podem prover acesso do fungo aos nutrientes do organismo, chegando mesmo a penetrar nas células do hospedeiro. O objetivo deste estudo foi avaliar a carga fúngica bucal e a secreção de enzimas histolíticas das espécies de *Candida albicans* isoladas de adultos saudáveis não-fumantes e fumantes . Cem adultos (42 não-fumantes e 58 fumantes) participaram do estudo. Os espécimes foram coletados do dorso da língua e da mucosa jugal (direita e esquerda). A identificação das espécies de *Candida* foi realizada utilizando o meio de cultura CHROMagar®Candida. A positividade do gênero *Candida* foi de 28,57% nos indivíduos não-fumantes e de 39,65% fumantes ($P = 0,254$). *C. albicans* foi a espécie mais prevalente nos dois grupos, sendo isolada em 78,57% nos não-fumantes e 72,41% nos fumantes. Outras espécies encontradas foram *C. parapsilosis, C. glabrata e C. tropicalis.* Dos indivíduos positivos foi selecionada aleatoriamente uma cepa de cada espécie, totalizando 43 cepas, que foram avaliadas quanto à produção das enzimas aspartil-protease (Sap), fosfolipase, condroitinase, esterase-lipase e hemolisina. A atividade enzimática foi maior no grupo dos não-fumantes para todas as enzimas estudadas, com diferença estatística significativa na atividade da enzima fosfolipase, sendo maior nas cepas de pacientes não-fumantes ($P = 0,013$). Concluímos que o tabagismo não afetou significativamente a positividade de *Candida spp.* e nem a atividade enzimática das cepas de *C. albicans*.

Palavras chave: *Candida albicans;* fumantes; enzimas; fosfolipase.

PÁGINA DE TÍTULO

FATORES DE VIRULÊNCIA DE CEPAS DE *Candida albicans* ISOLADAS DE FUMANTES E NÃO-FUMANTES

Ana Claudia Santos de Azevedo Izidoro, Edvaldo Antônio Ribeiro Rosa*, Maria Ângela Naval Machado

*Autor responsável pela correspondência

Endereço: Pontifícia Universidade Católica do Paraná

Laboratório de Estomatologia - CCBS

Rua Imaculada Conceição, nr 1155 – Prado Velho – Curitiba - Paraná.

CEP: 80 215 - 901

Telefone comercial: (041) 3271-1497

Fax: (041) 3271-1405

E-mail: edvaldo.rosa@pucpr.br

RESUMO

O cigarro de tabaco tem sido considerado como um fator predisponente para várias doenças, inclusive para a candidose bucal. Ele contem substâncias que podem ser degradadas por enzimas das espécies de *candida,* servindo como nutrientes. Por outro lado, a produção de enzimas histolíticas pelas espécies de *Candida* é um fator importante no desenvolvimento de doenças provocadas por fungos oportunistas. As enzimas podem prover acesso do fungo aos nutrientes do organismo, chegando mesmo a penetrar nas células do hospedeiro. O objetivo deste estudo foi avaliar a carga fúngica bucal e a secreção de enzimas histolíticas das espécies de *Candida albicans* isoladas de adultos saudáveis não-fumantes e fumantes . Cem adultos (42 não-fumantes e 58 fumantes) participaram do estudo. Os espécimes foram coletados do dorso da língua e da mucosa jugal (direita e esquerda). A identificação das espécies de *Candida* foi realizada utilizando o meio de cultura CHROMagar®Candida. A positividade do gênero *Candida* foi de 28,57% nos indivíduos não-fumantes e de 39,65% fumantes ($P = 0,254$). *C. albicans* foi a espécie mais prevalente nos dois grupos, sendo isolada em 78,57% nos não-fumantes e 72,41% nos fumantes. Outras espécies encontradas foram *C. parapsilosis, C. glabrata e C. tropicalis.* Dos indivíduos positivos foi selecionada aleatoriamente uma cepa de cada espécie, totalizando 43 cepas, que foram avaliadas quanto à produção das enzimas aspartil-protease (Sap), fosfolipase, condroitinase, esterase-lipase e hemolisina. A atividade enzimática foi maior no grupo dos não-fumantes para todas as enzimas estudadas, com diferença estatística significativa na atividade da enzima fosfolipase, sendo maior nas cepas de pacientes não-fumantes ($P = 0,013$). Concluímos que o tabagismo não afetou significativamente a positividade de *Candida spp.* e nem a atividade enzimática das cepas de *C. albicans*.

Palavras chave: *Candida albicans;* fumantes; enzimas; fosfolipase.

INTRODUÇÃO

C. albicans é um fungo dimórfico, que está presente em aproximadamente 50% dos indivíduos saudáveis (37). Além da *C. albicans*, outras espécies como *C. tropicalis, C. glabrata, C. krusei e C. parapsilosis* são capazes de competir com a microbiota bucal e se tornarem patogênicas.

Segundo Almeida & Scully (1), os principais fatores que dificultam o crescimento desses fungos no homem são a elevada temperatura e a imunidade celular. A virulência da *C. albicans* está associada com o aumento da produção de tubo germinativo pelas leveduras em baixas temperaturas. Leveduras que crescem em temperatura ambiente são mais resistentes à ação dos leucócitos polimorfonucleares (2). O desenvolvimento

de micélio pelas espécies de *Candida* ssp. (dimorfismo) favorece as infecções fúngicas em decorrência da variabilidade antigênica da superfície e do formato que propicia maior aderência, dificultando a ação fagocitária pelo sistema imune (23).

O tabagismo pode estar associado à predisposição às candidoses bucais (15, 31, 45). A nicotina no tabaco pode causar mudanças estruturais e funcionais nos queratinócitos (25). O efeito do fumo nas células epiteliais, reduz a atividade anti-*Candida* das células e pode aumentar a susceptibilidade à candidose orofaringeana (42), além de causar um espessamento na camada queratinizada do epitélio, o que pode contribuir para a maior colonização pela levedura (4, 37, 44).

Pacientes fumantes relatam recidiva das infecções por *Candida* após o término da terapia antifúngica, por outro lado, há evidências clínicas de que algumas infecções desaparecem apenas com a cessação do vício de fumar (15).

A produção de enzimas histolíticas é um fator importante no desenvolvimento de doenças provocadas por fungos oportunistas, sobretudo em isolados clínicos de *Candida* spp. As enzimas podem prover acesso do fungo aos nutrientes do organismo, chegando mesmo a penetrar nas células do hospedeiro, via estruturas tipo haustório (14). Quando a hidrólise dos substratos ou ação das proteínas extracelulares afeta a função e a viabilidade do hospedeiro, as enzimas podem ser consideradas fatores de virulência que contribuem para o estabelecimento da infecção (7, 22, 32).

O objetivo deste estudo foi avaliar a carga fúngica bucal e os perfis de virulência das cepas de *Candida albicans* entre indivíduos não-fumantes e fumantes. Para isto foram realizados os seguintes testes: contagem de diferentes espécies de *Candida*, produção de aspartil-proteases secretoras (Sap), de fosfolipases, de condroitinase, de esterase/lipase e de hemolisinas.

MATERIAL E MÉTODOS

A participação dos indivíduos foi condicionada à assinatura de um Termo de Consentimento Livre e Esclarecido, em concordância com as diretrizes do Comitê de Ética em Pesquisa da Pontifícia Universidade Católica do Paraná (PUCPR).

Foram avaliados 100 indivíduos, 42 não-fumantes e 58 fumantes, e considerados fumantes aqueles com consumo igual ou superior a cinco cigarros por dia há pelo menos um ano, e não-fumantes aqueles que relataram nunca terem fumado (29). Indivíduos que fumavam menos de cinco cigarros por dia foram excluídos da pesquisa. Cada indivíduo foi avaliado por um único avaliador que realizou a anamnese e o exame físico intrabucal, inspecionando as mucosas, tecidos moles e dentes. Indivíduos com manifestações clínicas de candidose bucal; relato de doenças sistêmicas na anamnese (diabetes ou hipertensão); mulheres grávidas; indivíduos que tivessem utilizado antibióticos nos três meses que precederam as coletas ou fazendo uso de medicação sialorredutora, foram excluídos da amostra. A média de idade dos indivíduos não-fumantes foi de 19,95 ± 2,13 anos e dos fumantes de 22,83 ± 5,75 anos, sendo em sua maioria jovens. A média de cigarros consumidos por dia foi 12,79 ± 5,73 e do tempo de uso em anos foi de 6,19 ± 5,10.

Amostras de raspado superficial (*swabbing*) do dorso da língua e da mucosa jugal dos indivíduos foram recolhidas, semeadas em placas contendo CHROMagar®*Candida* (CHROMagar Microbiology, Biomerieux, Paris, França) e incubadas a 37ºC, por 48 horas. Após a identificação fenotípica (24), as cepas foram transferidas para tubos de armazenamento contendo Ágar Sabouraud Dextrose (Difco Laboratories, Detroit, Mich., USA).

Um total de 43 cepas do gênero *Candida* (uma colônia/fenótipo por indivíduo) foram submetidas às provas de atividade enzimática. A cepa-padrão de *C. albicans* CBS562 proveniente do estoque do Laboratório de Estomatologia da PUCPR foi testada como controle. Os isolados foram crescidos em estufa a 37ºC em 5mL de Caldo Sabouraud Dextrose (Difco Laboratories, Detroit, Mich., USA). Após 24 horas as células foram recolhidas por centrifugação e lavadas três vezes com água destilada estéril. Os *pellets* foram ressuspendidos em água estéril até o equivalente ao tubo 10 da escala de MacFarland e 5 μL inoculados com o auxílio de discos de papel filtro esterilizados, em cinco pontos eqüidistantes, nos meios de cultura para detecção semi-quantitativa de aspartil-protease secretora (Sap), fosfolipases, condroitinase, esterase/lipase e hemolisinas. Todos os testes foram realizados em duplicata.

A secreção de Sap foi verificada seguindo o protocolo de Rüchel *et al.* (32), empregando a fração V da albumina bovina (Sigma Chem Co., St. Louis, Mo., USA) como substrato, Yeast Nitrogen Base (Difco Laboratories, Detroit, Mich., USA) e 2,5 mL de Protovit Plus® (Roche, São Paulo, Brasil) como co-fatores (22). A atividade enzimática foi evidenciada pela formação de um halo transparente ao redor da colônia dez dias após a inoculação. (10).

A atividade fosfolipásica foi pesquisada seguindo o método de Price *et al.*(28). Em 1.000 mL de água destilada estéril foram dissolvidos 10,0 g de peptona (Difco Laboratories, Detroit, Mich., USA), 30,0g de glucose, 57,3g de NaCl e 0,55g de $CaCl_2$ e 20,0g de Ágar (Oxoid, Fakola AG, Basel, Switzerland). O meio foi esterelizado em autoclave a 120ºC por 15 minutos e resfriado a uma temperatura de 55ºC. O *egg yolk* estéril sem telurito de potássio (Newprov Prod. Laborat., Inc., São José dos Pinhais, Paraná, Brasil) foi adicionado ao meio esterelizado. Após 4 dias da inoculação, as

placas foram observadas quanto à formação de uma zona de cor amarelada ao redor das colônias.

Para a pesquisa de condroitinase foi empregado o protocolo de Smith & Willett (41) modificado. O meio de cultura foi preparado usando-se neopeptona 10 g, glucose 40 g, Ágar noble 15 g (Sigma Chem Co., St. Louis, Mo., USA) e água destilada qsp 1.000 mL e autoclavado a 120ºC por 15 minutos. Após resfriamento a 55ºC, foi acrescentada uma solução de albumina bovina fração V a 10% e sulfato de condroitina a 4% (Sigma Chem Co., St. Louis, Mo., USA), dissolvidos em 10 mL de água destilada estéril e esterelizados em filtros de Millipore de 0,22 μm. O pH final foi ajustado entre 6,8 ± 0,2. O halo formado foi medido após 48 h de inoculação.

O teste de opacidade Tween® 80 (Sigma Chem Co., St. Louis, Mo., USA) foi utilizado para determinar a produção de esterase/lipase. O meio de cultura foi preparado com 15,0 g de SDA (Difco Laboratories, Detroit, Mich., USA) e 10 g $CaCl_2$, em 1.000 mL de água destilada (33). Após ser autoclavado, o meio foi resfriado a 50ºC e 10 mL de Tween®80 (Sigma) autoclavado foi adicionado. As inoculações permaneceram em estufa a 37ºC por sete dias consecutivos.

Para determinar a atividade hemolítica, as cepas foram semeadas em placas de ágar-sangue contendo 70 mL de sangue de ovelha fresco e desfibrinado (Newprov Prod. Laborat., Inc., São José dos Pinhais, Paraná, Brasil); 30 g de glucose (Sigma Chem Co., St. Louis, Mo., USA); qsp 1.000 mL de solução de 1% de Ágar-ágar (Oxoid, Fakola AG, Basel, Switzerland); pH final 5,6 (19). A formação do halo translúcido ao redor das colônias após 48 h de incubação a 37ºC indicou atividade hemolítica positiva.

Após os períodos de observação foram feitas as leituras do tamanho das colônias e dos halos formados por um único pesquisador calibrado, utilizando transiluminação e um paquímetro digital (Mitutoyo, 6" digital caliper, w/abs, Japão) e calculadas as

atividades enzimáticas. Estas atividades foram determinadas de acordo com a técnica de Price *et al.* (28), dividindo-se o diâmetro da colônia (DC) pelo diâmetro do halo formado (DH). Este resultado é denominado Pz, que representa numericamente a atividade enzimática da cepa testada. Como menores valores Pz indicam maior atividade enzimática e vice-versa, optou-se por subtrair tais valores de um (1-Pz) (26). Os dados assim obtidos foram analisados quanto à sua normalidade de distribuição pelo teste de Kolmogorov-Smirnov. Para aspartil-protease secretora, condroitinase e hemolisinas houve distribuição normal dos valores e foi utilizado o teste *t* de Student. Nas enzimas onde não houve distribuição normal, fosfolipase e esterase/lipase, foi utilizado o teste não-paramétrico U de Mann-Whitney.

A atividade enzimática foi analisada estatisticamente somente nos isolados de *C. albicans*, devido à inexpressiva obtenção de outras espécies.

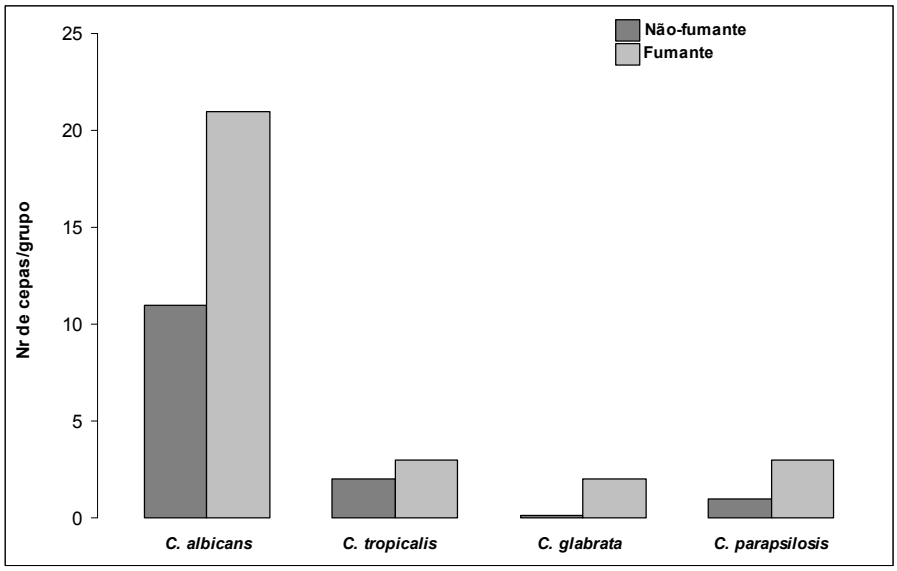
Além dos ensaios estatísticos, os valores 1-Pz também permitiram a classificação das cepas em zimotipos. Foram gerados dígitos a partir de um escore de atividade dado aos valores de Pz (7). Cepas não produtoras (Pz = 1,000) foram codificadas como 1, pois o diâmetro do halo foi considerado igual ao da colônia; cepas com atividade média ($Pz \leq 0,639$) receberam código 2; cepas com atividade elevada ($0,999 \leq Pz \geq 0,640$) receberam código 3 (26). Os zimotipos foram gerados seguindo a ordenação dos dígitos oriundos da secreção das enzimas aspartil-protease secretora, fosfolipases, condroitinase, esterase/lipase e hemolisinas. A zimotipagem nos forneceu uma visão geral da capacidade produtora de cada cepa, associando as cinco enzimas pesquisadas.

RESULTADOS

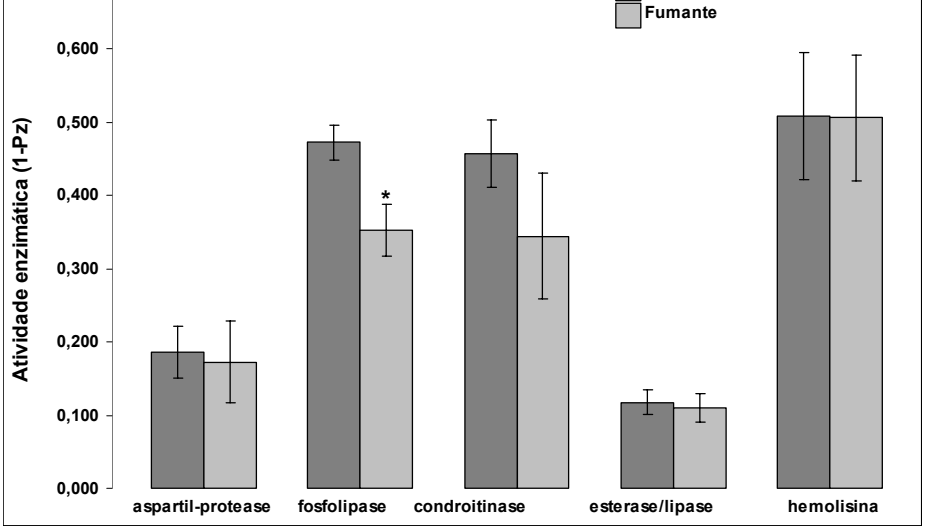
Isolamentos positivos de *Candida spp.* foram obtidos em 35 dos 100 indivíduos examinados, sendo 12/42 (28,57%) do grupo dos não-fumantes e 23/58 (39,65%) do grupo de fumantes, não havendo diferença estatística significante entre os grupos (P = 0,254).

Foram isoladas duas cepas de *C. tropicalis* 2/14 (14,28%) e uma de *C. parapsilosis* 1/14 (7,14%) dos indivíduos não-fumantes. Nos fumantes foram encontradas três cepas de *C. tropicalis* 3/29 (10,35%), duas de *C. glabrata* 2/29 (6,89%) e três de *C. parapsilosis* 3/29 (10,34%). O pequeno número isolado destas espécies não possibilitou análise estatística.

C. albicans foi prevalente em ambos os grupos, ocorrendo em 11/14 (78,57%) dos não-fumantes e 21/29 (72,41%) dos fumantes, não havendo diferença estatística significante entre eles (P = 0,705) quando o teste t de Student foi aplicado. Não houve predomínio de colonização de nenhuma das espécies encontradas em ambos os grupos (Gráfico 1).



Graf. 1. Distribuição das espécies de *Candida* isoladas de não-fumantes (n=14) e fumantes.



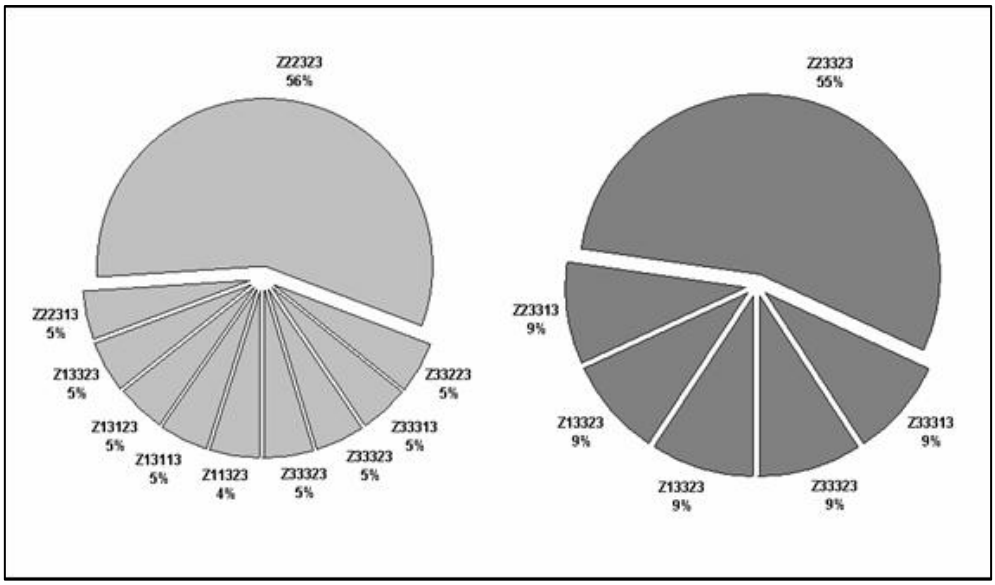
Graf. 2. Atividade das cepas de *C. albicans* isoladas de não-fumantes (n=11) e fumantes (n=21). * Teste U de Mann-Whitney (p

Das cepas de *C. albicans* isoladas de não-fumantes, 7/11 (63,63%) delas apresentaram atividade enzimática média para aspartil-protease, 2/11 (18,18%) apresentaram atividade elevada e em 2/11 (18,18%) não foi detectada atividade. Neste grupo, 11/11 (100%) das cepas apresentaram atividade elevada de fosfolipase, condroitinase e hemolisina. A atividade enzimática de esterase/lipase foi média em 9/11 (81,81%) cepas, e ausente em 2/11 (18,18%) dos isolados (Gráfico 2). A compilação dos escores de atividade enzimática das cepas de não-fumantes permitiu a obtenção de cinco zimotipos, sendo o Z32232 o mais prevalente.

Nas cepas isoladas de fumantes houve maior variação de atividade enzimática, sendo encontrados dez zimotipos. A aspartil-protease teve atividade elevada em 3/21 (14,28%) das cepas, assim como outras 3/21 não secretaram a enzima. Em 15/21 (71,42%) a atividade foi considerada média. Apenas uma 1/21 (4,76%) cepa não secretou fosfolipase, outras 2/21 (9,52%) apresentaram atividade média e 19/21 (90,47%) foram consideradas fortemente produtoras. Houve diferença estatística significante na produção desta enzima entre não-fumantes e fumantes (P = 0,013). Para condroitinase, 17/21 (80,95%) cepas apresentaram atividade elevada, 2/21 (9,52%) cepas apresentaram atividade média e as 2/21 cepas restantes não tiveram atividade detectável. Nenhuma cepa apresentou atividade elevada de esterase/lipase neste grupo.

A atividade foi média em 19/21 (90,47%) das cepas e ausente em 3/21 (14,28%) dos isolados. Na produção de hemolisina apenas uma cepa apresentou atividade média (4,76%), enquanto 20/21 (95,23%) apresentaram atividade elevada (Gráfico 2). O zimotipo predominante neste grupo foi o Z22323.

A cepa-padrão CBS562 apresentou o zimotipo coincidente com o mais encontrado nos fumantes, o Z23323, com produção de todas as cinco enzimas pesquisadas, tendo produção elevada em três delas (Gráfico 3).



Graf. 3. Distribuição dos zimotipos das cepas de *C. albicans* isoladas de não-fumantes (n=11) e fumantes (n=21).

DISCUSSÃO

Embora muitos artigos tenham sido publicados sobre a secreção de enzimas como fatores de virulência de *C. albicans* (6, 13, 17, 35, 36) até o nosso conhecimento, nenhum estudo anterior avaliou a atividade enzimática da espécie, ou do gênero, obtida de indivíduos saudáveis não-fumantes e fumantes.

No presente estudo, 28,57 % dos não-fumantes apresentou colonização por *Candida* spp. contra 39,65 % dos fumantes (p=0,254). Esses resultados são inferiores aos publicados por Rassol *et al*. (29), que isolaram cepas em 43% dos não-fumantes e 57% dos fumantes, embora estes autores também não tenham encontrado diferença estatística em os grupos. Em 1988, Odds (appud Scully) revisou 32 artigos sobre positividade de *Candida* spp. na boca de indivíduos saudáveis, e relatou que a média de positividade foi de 34,40%.

Em nosso estudo, *C. albicans* foi a espécie mais prevalente, em concordância com outros autores (22, 29, 37), sendo encontrada em 78,57% dos não-fumantes e 72,41% dos fumantes, quando esses se apresentavam colonizados. Embora tenha sido encontrado um percentual maior de isolamento dessa espécie no grupo dos não-fumantes, não há diferença estatística entre os grupos.

A positividade de produção de aspartil-proteases (Saps) pelas cepas encontradas no presente estudo está de acordo com os estudos de Bramono *et al*. (6), que encontraram atividade desta enzima nas cepas de *C. albicans, C. tropicalis, C. parapsilosis, C. glabrata, C. krusei e C. guilliermondii.* Mas, difere do encontrado por Dóstal *et al.* (10), que não encontraram atividade em cepas de *C. glabrata*.

Com relação às fosfolipases, não foi encontrada qualquer atividade nas cepas de *C. glabrata* e *C. parapsilosis*, o que contraria os achados de outros autores (11, 38), que

afirmam que estas espécies são capazes de produzir tais enzimas. Tal fato pode ter ocorrido devido ao número reduzido de isolamentos dessa espécie nos indivíduos arrolados neste estudo.

Quando a secreção dessas fosfolipases foi avaliada em isolados de *C. albicans* provenientes de não-fumantes, detectamos uma significante elevação de atividade (P = 0,013) em relação aos isolados de fumantes. Por se tratar de uma enzima que parece estar diretamente relacionada com a virulência desta espécie, era esperado que no grupo dos fumantes esta secreção fosse maior. Tal fato pode ser explicado em função da seleção aleatória das colônias que deram origem aos isolados analisados. Como foi selecionada apenas uma colônia de *C. albicans* por indivíduo, podem ter sido excluídas do estudo outras cepas, com maiores atividades enzimáticas.

Ibrahim *et al.* (13) reportaram que cepas comensais com baixa produção de fosfolipase são capazes de colonizar a mucosa, sugerindo que a secreção destas enzimas não é um requisito primordial para a patogenicidade de *C. albicans*, opinião compartilhada por Mayser *et al.* (21). Como os indivíduos aqui analisados não apresentavam quaisquer sinais clínicos de candidose bucal, é razoável supor que essa maior secreção de fosfolipases por cepas de não-fumantes não deva ter maiores implicações na colonização.

A totalidade das cepas de *C. albicans* do grupo não-fumantes produziu condroitinase, em comparação com 90,47% produzidos por esta espécie no grupo dos fumantes. Das espécies "*não-albicans*" apenas *C. tropicalis* e uma cepa de *C. parapsilosis* produziram condroitinase. Ainda, as duas cepas de *C. glabrata* isoladas não produziram condroitinase. Este resultado está de acordo com Shimizu *et al*. (39) que observaram a produção da enzima em 97,8% das cepas de *C. albicans*, sendo esta produção seguida por *C. tropicalis, C. guilliermondii, C. parapsilosis* e *C. krusei*, e com

Chaffin *et al.* (9) que afirmam que hialuronidase e condroitinase podem ser produzidas por *C. albicans, C. tropicalis, C. guilliermondii, C. parapsilosis,* e *C. krusei*, mas em uma pequena proporção dos isolados.

No presente estudo foi avaliado também a secreção de esterase/lipase. Todas as espécies foram capazes de secretar esta enzima; porém, em diferentes proporções. Este achado está de acordo com as pesquisas de outros autores (6, 9, 33, 43). O papel exato da atividade lipolítica na patogenicidade das cepas ainda não foi esclarecido.

A atividade das hemolisinas está associada à capacidade que as cepas têm em destruir hemácias e utilizar o ferro como nutriente. Nossos resultados mostraram que todas as espécies encontradas produziram hemolisinas, em concordância com os estudos de Luo *et al.* (19), sendo fortemente produtoras na maioria das cepas.

Dentre as cepas de *C. albicans* examinadas, nenhuma apresentou elevados níveis de secreção para as cinco enzimas de forma concomitante, i.e. nenhuma apresentou valor 1-Pz maior que 0,640 para as cinco enzimas estudadas. Isto está em concordância com o estudo de Bramono *et al*. (6), que pesquisaram a secreção de aspartil-protease, lipase e α-glucosidase em cepas de *C. albicans*, e nenhuma exibiu altos níveis de atividade para as três enzimas simultaneamente.

Tem sido sugerido que o cigarro pode levar à alterações localizadas no epitélio que permitem a colonização por *Candida* (3, 25). Williams *et al*. (44) mostraram que uma maior queratinização favorece a aderência de *C. albicans*. Ainda, Barret-Bee *et al*. (5) propuseram que a fosfolipase teria um importante papel nessa aderência; proposição não compartilhada por Ghannoum *et al*. (12). Na mesma direção, Ray & Payne (30) mostraram que blastoporos de *C. albicans* podem degradar a queratina celular utilizando-se de proteases ácidas e Jayatilake *et al*. (14) propuseram que as fosfolipases

também podem ser participar desta destruição, o que, em última instância, favoreceria a colonização.

A despeito dos argumentos acima, talvez a menor secreção de enzimas por cepas obtidas junto a fumantes se deva a uma seleção clonal determinada pela qualidade da saliva. Como as Saps (8) e as fosfolipases (20, 34) são usualmente ativas somente em meios ácidos e já foi mostrado que fumantes apresentam saliva mais ácida e com menor capacidade tamponante que não-fumantes (17, 18, 27, 40), é razoável supor que no grupo dos fumantes não seja necessária uma alta taxa de secreção para que as cepas possam conduzir sua colonização.

De acordo com a literatura disponível, fumantes podem ter maior predisposição à colonização por *Candida spp.* devido à indução de um aumento na queratinização do epitélio (4, 25, 44) ; à diminuição da atividade dos leucócitos, do exudato gengival (42) e dos níveis de imunoglobulina A (42); sendo relatados os efeitos do fumo no indivíduo, mas não nos microorganismos.

Mais estudos são necessários para identificar o efeito, se é que existe, do cigarro sobre as espécies de *Candida* que fazem parte da microbiota bucal humana.

CONCLUSÃO

Concluímos com nosso estudo que a carga fúngica bucal não difere entre não-fumantes e fumantes. O perfil de virulência não diferiu entre não-fumantes e fumantes, exceto para a secreção das fosfolipases, pois houve uma menor secreção destas enzimas nas cepas de *Candida albicans* de indivíduos fumantes.

REFERÊNCIAS

1. **Almeida, O. P. and C. Scully.** 2002. Fungal infections of the mouth. Braz J Oral Sci. **1:** 19-26.
2. **Antley, P. P. and Hazen K. C.** 1988. Role of yeast cell growth temperature on *Candida albicans* virulence in mice. Infect. Immun. **56:** 2884-2890.
3. **Arendorf, T. M. and D. M Walker.** 1984. Tobacco smoking and denture wearing as local aetiological factors in median rhomboid glossitis. *Int J Oral Surg*. **13:** 411-415.
4. **Arrendondo, J., Nguyen V. T. and Chernyavsky A. I.** 2001. A Receptor-mediated mechanism of nicotine toxicity in oral candidal leukoplakia. Br. Dent. J. **155:** 340-343.
5. **Barrett-Bee K., Y. Hayes, R. G. Wilson and J. F. Ryley.** 1985. A comparison of phospholipase activity, cellular adherence and pathogenicity of yeasts. J Gen Microbiol. **131:** 1217-1221.
6. **Bramono K., M. Yamazaki, R. Tsuboi, H. Ogawa.** 2006. Comparison of proteinase, lipase and alpha-glucosidade activities from the clinical isolates of *Candida* species. Jpn. J. Infect. Dis. **59:** 73-76.
7. **Candido, R. C., C. Azevedo, R. V. P. Azevedo, M. C. Komesu.** 2000. Enzimotipagem de espécies do gênero *Candida* isoladas da cavidade bucal. Rev. Soc. Bras. Med. Trop. **33:** 437-442.
8. **Cassone A., F. De Bernardis, F. Mondello, T. Ceddia and L. Agatensi.** 1987. Evidence for a correlation between proteinase secretion and vulvovaginal candidosis. J. Infect. Dis. **156:** 777-783.

9. **Chaffin, W. L., J. L. López-Ribot, D. G. Casanova and J. P. Martinez.** 1998. Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. Microbiol. Mol. Biol. Rev. **62:** 130-180.
10. **Dostál, J., P. Hamal, L. Pavlíčková, M. Souček, T. Ruml, I. Pichová and O. Hrušková-Heidingsfeldová.** 2003. Simple method for screening *Candida* species isolates for the presence of secreted proteinases: a tool for the prediction of successful inhibitory treatment. J. Clin. Microbiol. **41:** 712-716.
11. **Ghannoum, M. A.** 2000. Potential role of phospholipases in virulence and fungal pathogenesis. Clin. Microbiol. Rev**. 13:** 122-143.
12. **Ghannoum M. A., S. G. Filler, A. S. Ibrahim, Y. Fu and J. E. Edwards Jr.** 1992. Modulation of interactions of *Candida albicans* and endothelial cells by fluconazole and amphotericin B. Antimicrob. Agents Chemother. **36:** 2239-2244.
13. **Ibrahim A. S., F. Mirbod, S. G. Filler, Y. Banno, G. T. Cole, Y. Kitajima, J. E. Edwards Jr., Y. Nozawa and M. A. Ghannoum.** 1995. Evidence implicating phospholipase as a virulence factor of *Candida albicans.* Infect. Immun. **63:** 1993-1998.
14. **Jayatilake J. A., Y. H. Samaranayake and L. P. Samaranayake.** 2005. An ultrastructural and a cytochemical study of candidal invasion of reconstituted human oral epithelium. J. Oral Pathol. Med. **34:** 240-246.
15. **Johnson, N.W. and C. A. Bain.** 2000. Tobacco and oral disease. British Dental Journal. **189:** 200-206.
16. **Kivela J., S. Parkkila, J. Metteri, A. K. Parkkila, A. Toivanen and H. Rajaniemi.** 1997. Salivary carbonic anhydrase VI concentration and its relation

to basic characteristics of saliva in young men. Acta Physiol. Scand. **161:** 221-225.

17. **Koga-Ito, C.Y., J. P. Lyon, V. Vidotto and M. A. de Resende.** 2006. Virulence factors and antifungal susceptibility of *Candida albicans* isolates from candidoses patients and control individuals. Mycopathologia. **161:** 219-223.
18. **Liede K. E., J. K. Haukka, J. H. Hietanen, M. H. Mattila, H. Ronka and T. Sorsa.** 1999. The association between smoking cessation and periodontal status and salivary proteinase levels. J. Periodontol. **70:** 1361-1368.
19. **Luo, G., L. P. Samaranayake and J. Y. Y. Yan.** 2001. *Candida* species exhibit differential in vitro hemolytics activities. J. Clin. Microbiol. **39:** 2971-2974.
20. **Lyon J. P. and M. A. de Resende.** 2006. Correlation between adhesion, enzyme production, and susceptibility to fluconazole in *Candida albicans* obtained from denture wearers. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. **102:** 632-638.
21. **Mayser, P., S. Laabs, K-U Heuer and K. Gründer.** 1996. Dection of extracelular phospholipase activity in *Candida albicans* and *Rhodotorula rubra.* Mycopathologia. **135:** 149-155.
22. **Menezes, E. A., M. S. Cavalcante, R. B. Farias, A. B. Teixeira, F. G. Pinheiro, B. P. Bezerra, J. C. N. Torres and F. A. Cunha.** 2005. Frequency and enzymatic activity of *Candida albicans* isolated from the buccal mucosa of children of a day-care center of the city hall of Fortaleza, Ceará, Brazil. J. Bras. Patol. Med. Lab. **41:** 9-13.
23. **Molero, G., R. Diez-Orejas, F. Navarro-Garcia, L. Monteoliva, J. Pla, C. Gil, M. Sanchez-Perez and C. Nombela.** 1998. *Candida albicans*: genetics, dimorphism and pathogenicity. Internatl. Microbiol. **1:** 95–106.

24. **Odds, F.C. and R. Bernaerts.** 1994. CHROMagar *Candida*, a new differential isolation medium for presumptive identification of clinically important *Candida* species. J. Clin. Microbiol. **32:** 1923-1929.

25. **Orellana-Bustos A.I., I. L. Espinosa-Santander, E. Franco-Martinez, N. Lobos-Jaimes-Freyre and A. V. Ortega-Pinto.** 2004. Evaluation of keratinization and AgNORs count in exfoliative cytology of normal oral mucosa from smokers and non-smokers. Med. Oral. **9:** 197-203.

26. **Ozkan, S., F. Kaynak, A. Kalkanci, U. Abbasoglu and S. Kustimur.** 2005. Slime production and proteinase activity of *Candida* species isolated from blood samples and the comparison of these activities with minimum inhibitory concentration values of antifungal agents. Mem. Inst. Oswaldo Cruz. **100:** 319-324.

27. **Parvinen, T.** 1984. Stimulated salivary flow rate, pH and lactobacillus and yeast concentrations in non-smokers and smokers. Scand. J. Dent. Res. **92:** 315-318.

28. **Price M. F., I. D. Wilkinson and L. O. Gentry.** 1982. Plate method for detection of phospholipase activity in *Candida albicans*. Sabouraudia. **20:** 7-14.

29. **Rassol, S., C. H. Siar and K. P. Ng.** 2005. Oral candidal species among smokers and non-smokers. JCPSP. **15 :** 679-682.

30. **Ray, T.L. and C.D. Payne.** 1988. Scanning electron microscopy of epidermal adherence and cavitation in murine candidiasis: a role for *Candida* acid proteinase. Infect. Immun. **56:** 1942-1949.

31. **Rindum J.L., A. Stenderup and P. Holmstrup.** 1994. Identification of *Candida albicans* types related to healthy and pathological oral mucosa. J. Oral Pathol. Med. **23:** 406-412.

32. **Ruchel, J., R. Tegeler and M. A. Trost.** 1982. Comparison of secretory proteinases from different strains of *Candida albicans.* Sabouradia. **20:** 233-244.

33. **Rudek, W.** 1978. Esterase activity in *Candida* species. J. Clin. Microbiol. **8:** 756-759.

34. **Samaranayake L. P., J. M. Raeside and T. W. MacFarlane.** 1984. Factors affecting the phospholipase activity of *Candida* species in vitro. Sabouraudia. **22:** 201-207.

35. **Samaranayake, Y. H., R. S. Dassanayake, J. A. M. S. Jaytilake, B. P. K. Cheung, J. Y. Y. Yan, K. W. S. Yeung and L.P. Samaranayake**. 2005. Phospholipase B enzyme expression is not associated with other virulence attributes in *Candida albicans* isolates from patients with human immunodeficiency virus infection. J. Med. Microbiol. **54:** 583-593.

36. **Schaller, M., C. Borelli, H. C. Korting and B. Hube.** 2005. Hydrolytic enzymes as virulence factors of *Candida Albicans*. Mycoses. **48:** 365-377.

37. **Scully, C., M. El-Kabir and L. P. Samaranayake.** 1994. *Candida* and oral candidosis: a review. Crit. Rev. Oral Biol. Med. **5:** 125-157.

38. **Serda-Kantarcioğlu A. and A. Yücel.** 2002. Phospholipase and protease activity in clinical *Candida* isolates with reference to the sources of strains. Mycoses. **45:** 160-165.

39. **Shimizu M. T., A. O. Jorge, C. S. Unterkircher, V. Fantinato and C. R. Paula.** 1995. Hyaluronidase and chondroitin sulphatase production by different species of *Candida*. J. Med. Vet. Mycol. **33:** 27-31.

40. **Sitheeque M. A. M. and L. P. Samaranayake.** 2003. Chronic hyperplastic candidosis/candidiasis (candidal leukoplakia). Crit. Rev. Oral Biol. Med. **14:** 253-267.

41. **Smith R. F. and N. P. Willett.** 1968. Rapid plate method for screening hyaluronidase and chondroitin sulfatase producing microorganisms. Appl. Microbiol. **16:** 1434-1436.

42. **Soysa, N. S. and A. N. B. Ellepola.** 2005. The impact of cigarette/tobacco smoking on oral candidosis: an overview. Oral Diseases. **11:** 268-273.

43. **Tsuboi, R.**, **H. Komatsuzaki and H. Ogawa.** 1996. Induction of an extracellular esterase from *Candida albicans* and some of its properties. Infect. Immun. **64:** 2936-2940.

44. **Williams D. W., R. Walker, M. A. Lewis, R. T. Allison and A. J. Potts.** 1999. Adherence of *Candida albicans* to oral epithelial cells differentiated by Papanicolaou staining. J Clin Pathol. **52:** 529-531.

45. **Willis A. M., W. A. Coulter, C. R. Fulton, J. R. Hayes, P. M. Bell and P. J. Lamey.** 1999. Oral candidal carriage and infection in insulin-treated diabetic patients. Diabet Med. **16:** 675-679.

5. ARTIGO EM INGLÊS

TITLE PAGE

VIRULENCE FACTORS OF *Candida albicans* STRAINS ISOLATED FROM SMOKERS AND NON-SMOKERS

Ana Claudia Santos de Azevedo Izidoro, Edvaldo Antônio Ribeiro Rosa*, Maria Ângela Naval Machado

* Corresponding author.

Mailing address: Pontifícia Universidade Católica do Paraná

Laboratório de estomatologia - CCBS

Imaculada Conceição, 1155 – Prado Velho – Curitiba – Paraná - Brazil

ZIP code: 80 215 - 901

Business phone: 055 041 3271-1497

Fax number: 055 041 3271-1405

E-mail:edvaldo.rosa@pucpr.br

ABSTRACT

The cigarette smoke contains substances that can be degraded by enzymes of *Candida* species, been used like nutrients and converted to carcinogen end products. The purpose of this study was evaluated the carriage of *Candida* species and analyse the extracellular proteolytic activities of *Candida* isolates from healthy adults non-smokers and smokers. One hundred adults (42 non-smokers and 58 smokers) were studied. The samples were collected from intra oral swabs and smears from dorsum of tongue, right and left jugal mucosa each. The identification of species of *Candida* was made using the culture medium CHROMagar®Candida. The carriage of the gender *Candida* was 28,57% in non-smokers individuals and 39,65% in smokers (P = 0,254). *Candida albicans* was the most prevalent one between the two groups, being isolated in 78.57% in the non-smokers, and 72.41% in the smokers. Other species found were *C. parapsilosis, C. glabrata*, and *C. tropicalis*. From the positive individuals was random one strain of *C. albicans*, comprised 32 strains that were evaluated regarding the proteolytic activity of the following enzymes: secreted aspartyl-protease (Sap), phospholipase, chondroitinase, esterase-lipase, and haemolysin. Regarding the hydrolytic enzymes, there was bigger enzymatic activity in the group of non-smokers, in all studied enzymes, with significant statistical difference between both groups in the activity of phospholipase, that was bigger in the strains of non-smokers (P = 0,013). The results obtained in this study have shown that the smoking do not alter significantly the carriage of *Candida* spp., neither the enzymatic activity of *C. albicans* strains, except the activity of phospholipase.

Keywords: *Candida albicans*, smokers, enzymes, phospholipase.

INTRODUCTION

Candida albicans is present in the inoffensive form in the oral cavity of 50% of healthy people (37). Besides *C. albicans*, other species such *C. tropicalis, C. glabrata, C. krusei*, and *C. parapsilosis* are able to compete with the oral microbiota and become pathogenic. These forms of candidosis are mycotic infections relatively common and well known, however, not completely understood (22).

According to Almeida & Scully, (1) the main obstacles for these fungi to grown in humans are the high temperature and the cellular immunity. The virulence of *C. albicans* is associated to the increase of the production of the germinative tube by yeasts under low temperatures. Yeasts that grow under room temperature are more resistant to the actions of polymorph nuclear leukocytes (2). The development of mycelium by strains of *Candida* (dimorphism) also favours the infection as result of the antigenic variability of the surface and of the mycelial format that propitiates more adherence hindering the phagocytary action by the immune system (23).

The smoking is also associated to the predisposition to the oral candidosis (15, 31, 42, 45). The nicotine in the tobacco may cause structural and functional changes in the keratinocytes (25). The effects of tobacco on the epithelial cells reduces the anti-*Candida* activity of cells and may increase the susceptibility to oropharyngeal candidosis (42), and can cause thickening of the keratinised layer of the epithelium, that may contribute for a more pronounced colonization by yeast (37, 44).

Smoking subjects report recurrence of infections caused by *Candida* after the end of antifungal therapy; on the other hand, there are clinic evidences that some infections disappeared after ceasing the smoking habit (15).

The production of histolytic enzymes is an important factor in the development of diseases caused by opportunistic fungi, especially in clinic isolates of *Candida* spp. The enzymes may promote the access of the fungus to the nutrients of the organism, even penetrating the cells of the host through haustorium-like structures (14). When the hydrolysis of the substrata or the action of the extracellular proteins affects the function and viability of the host, the enzymes may be considered factors of virulence that contribute for the establishment of infection (7, 22, 32).

This study was carried aiming at assessment of the carriage of oral *Candida* species and the profiles of virulence of the strains of *Candida* among the smoking and non-smoking populations. Thus, there were performed the following tests: counting of the different species of *Candida*, productions of secreted aspartyl-proteases (Saps), phospholipases, chondroitinase, esterase/lipase, and of hemolysins.

MATERIAL AND METHODS

The participation of individuals was conditioned to the agreement of term of free and aware consentient with accordance to policies of the Research Ethics Committee of the *Pontifical Catholic University of Paraná* (*PUCPR*).

There were examined 100 individuals, 42 non-smoking and 58 smoking and there was considered chronic smoker that individual whose daily consumption has been equal or superior to five cigarettes for, at least, one year, and considered non-smoker that one who has never smoked before (29). Individuals whose daily consumption was lower to five cigarettes per day, were excluded of the study. Each individual was evaluated by only one professional who performed the anamnesis and a physical exam, survey of the

health conditions of the mucosa and other soft tissues as well as the teeth. Individuals with clinical manifestations of oral candidosis; relates of systemic diseases like diabetes, hypertension; pregnancy; or individuals used of antibiotics in the last three months before the collects, or using of sialoreductor drugs were excluding conditions of the sample. The medium age of the non-smokers individuals was 19,95 ± 2,13 years and of the smokers was 22,83 ± 5,75 years.

Sample of superficial scrapings (*swabbing*) of the back of the tong and jugal mucosa of subjects were taken, inoculated in dishes containing CROMAgar©Candida (CHROMagar Microbiology, Biomerieux, Paris, France) and incubated at 37ºC, for 48 hours. After the phenotypic identification (24), the strains were transferred to storing tubes containing Sabouraud Dextrose Agar (Difco Laboratories, Detroit, Mich., USA).

A total of 43 strains from the genus *Candida* (a colony/phenotype from each individual) which were submitted to checks of enzymatic activity. There were also tested the type-strain of *C. albicans* CBS562 from the archive of *Laboratory of Stomatology* of *PUCPR*. The isolates were grown at 37ºC in 5mL of Sabouraud Dextrose Broth (Difco Laboratories, Detroit, Mich., USA). After 24 hours, the cells collect through centrifugation and washed three times with sterile distilled water. The pellets were resuspended in sterile water until the equivalent to the tube #10 of the scale of MacFarland and inoculated, in equidistant points, in the culture media for the semi-quantitative detection of secreted aspartyl-protease (Sap), phospholipases, chondroitinase, esterase/lipase, and haemolysins. All the tests were made in duplicate.

The secretion of Sap was verified following the protocol of Ruchel *et al.* (32), employing the fraction V of bovine albumin (Sigma Chem Co., St. Louis, Mo., USA) as substratum, Yeast Nitrogen Base (Difco Laboratories, Detroit, Mich., USA) and 2.5ml of Protovit®Plus (Roche Laboratories, São Paulo, Brazil) as cofactors (22). The

enzymatic activity was evidenced by the formation of a translucent around the colony, 10 days after the inoculation. (10).

The phospholipasic activity was researched according to the method of Price *et al.* (28). In 1.000 mL of sterile distilled water, there were dissolved 10.0g of peptone (Difco Laboratories, Detroit, Mich., USA) 30.0g of glucose, 57.3g of NaCl, 0.55g $CaCl_2$, and 20.0g of agar (Oxoid, Fakola AG, Basel, Switzerland). The medium was sterilized in autoclave at 120ºC for 15 minutes and cooled to a temperature of 55ºC. The sterile egg yolk without potassium tellurite (Newprov Prod. Laborat., Inc., São José dos Pinhais, Paraná, Brazil) was added to the sterilized medium. After 4 days of inoculation, the dishes were observed regarding the formation of an area of yellow colour around the colonies.

Concerning the research of chondroitinase, there was employed the modified protocol of Smith & Willett (41). The culture medium was prepared using neopeptone 10g, glucose 40g, agar noble 15g (Sigma Chem Co., St. Louis, Mo., USA) and distilled water till 1.000mL and autoclaved at 120ºC for 15 minutes. After cooling to 55ºC, there was added 10mL of sterile solution of 10% fraction V bovine albumin plus 4% chondroitin sulphate (Sigma). The final pH was adjusted among 6.8 ± 0.2. The inoculations were kept in stove for 48 hours.

The Tween®80 (Sigma) opacity test was used for determining the production of esterase/lipase. The medium of culture was prepared with 15.0g of SDA (Difco) and 10g $CaCl_2$, in 1.000 mL of distilled water (33). After autoclaved, the medium was cooled to 50ºC and 10mL of Tween®80 autoclaved (Sigma) were added. The inoculations were kept in stove for seven consecutive days.

For determining the haemolytic activity, the strains were inoculated in dishes of Blood-Agar with 70 mL of fresh and defibrinated blood of sheep (Newprov); 30g of

glucose (Sigma); till 1.000 mL of 1% Agar-agar solution (Oxoid); final pH 5,6 (19). The formation of a translucent halo around the colonies after 48 hours of incubation has pointed positive haemolytic activity.

After the daily observation, there were performed the reading of the size of the colonies and size of halos formed for each enzyme by unique observer using transillumination and a digital caliper (Mitutoyo, Japan) and calculated the enzymatic activities. These enzymatic activities (Pz) were determined according to the technique of Price *et al.* (28), through the ratio among the diameter of the colony (CD) and the diameter of the formed halo (HD). This result is denominated Pz, and represents the enzymatic activity of each strain tested. As little values of Pz points a more expressive enzymatic activity and vice-versa, there was opted for subtracting such values from 1 (1-Pz) (26). The data therefore obtained were analysed with regard to their distribution by the test of Kolmogorov-Smirnov. For secreted aspartyl-protease, chondroitinase, and haemolysins, there was a normal distribution of the values, and there was used the Student's *t* test. In the enzymes, which did not present normal distribution (phospholipase and esterase/lipase), there was used the non-parametric Mann-Whitney *U* test.

The enzymatic activity was analysed only in isolates of *C. albicans* due to the inexpressive obtaining of other species.

Besides the statistic assays, the values of 1-Pz also allowed the classification of strains in zymotypes. Each digit was generated from a score of activity (7). Non-producer strains (Pz = 1.000) were codified as 1, besides the diameter of the formed halo was considered the same of the diameter of the colony; strains with intermediary activity ($Pz \leq 0{,}639$) received code 2; strains with high enzymatic activity ($0{,}999 \leq Pz \geq 0.640$) received code 3 (26). The zymotypes were generated following the ordination of

the digits resultant of the secreted aspartyl-protease, phospholipases, chondroitinase, esterase/lipase, and haemolysins. The zimotyping gave us an overall of the enzymatic activity that each strain associated the activity of the five enzymes studied.

RESULTS

Positive isolations of *Candida* were obtained in 43 out of the 100 collected sampled, being 12/42 (28,57%) from the check group and 29/58 (39,65%) from the group of smokers, don't having statistic difference among the groups (P = 0,254).

C. albicans was the most prevalent in both groups, occurring in 11/14 (78.57%) of non-smokers and 21/29 (72.42%) of smokers, not presenting statistic difference among the groups (P = 0.705) when the Student's t test was performed. Regarding the non-*albicans* species, there was no colonial predominance of any species in either group, as observed in graphic 1.

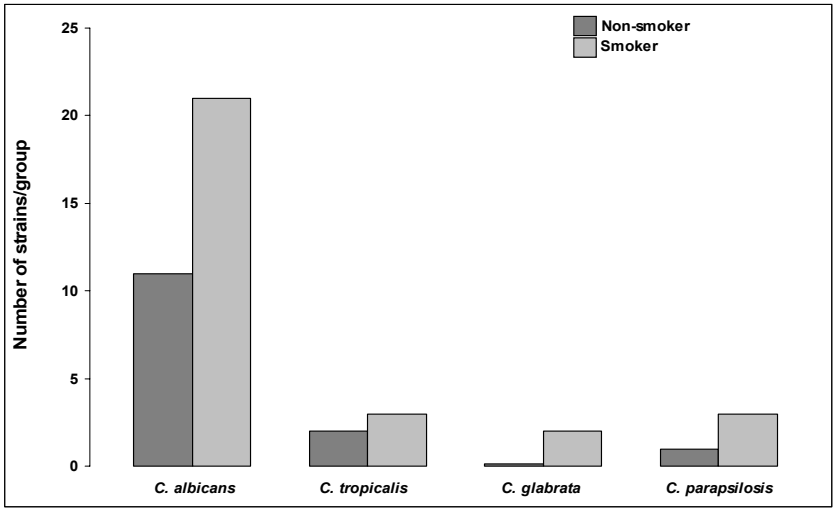


FIG. 1. Distribution of yeast species isolated from non-smokers (n = 14) and smokers (n = 29). No disparities were found when the Test for Differences Between Two Proportions was employed.

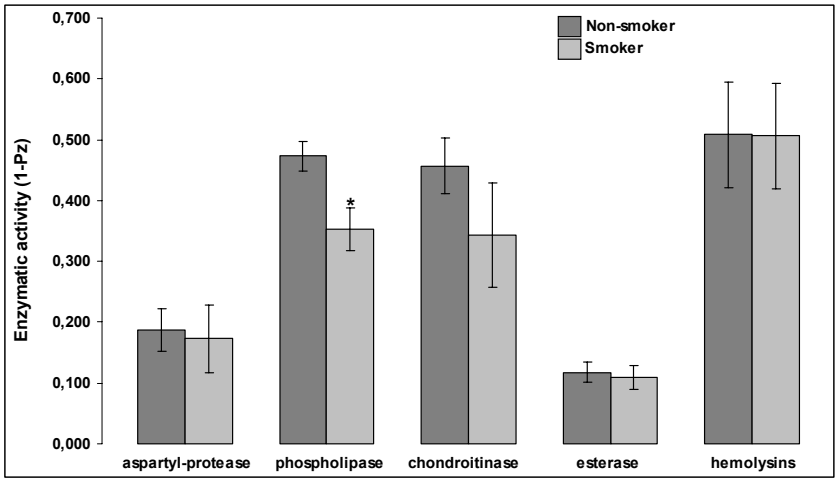


FIG. 2. Overall view of extracellular enzymatic activity of *C. albicans* strains isolated from non-smokers (n = 11) and smokers (n = 21). * Mann-Whitney U test; $P = 0.0131$.

From the strains of *C. albicans* isolated from non-smokers, 7/11 (63,64%) of them have presented mean enzymatic activity for secreted aspartyl-protease, 2/11 (18.18%) have presented high activity, and in 2/11 (18.18%), there was not detected activity. In this group, 11/11 (100%) of the strains have presented high activity of phospholipase, chondroitinase, and haemolysin. The enzymatic activity of esterase/lipase was medium in 9/11 (81.81%) strains and absent in 2/11 (18.18%) of the isolates. (Graphic 2).The compilation of scores of enzymatic activity allowed the obtaining of six zymotypes, being the Z23323 the most prevalent one.

In the strains isolated from smokers, there was a larger variation of the enzymatic activity, being found ten zymotypes (Figure 3). Secreted aspartyl-protease presented high activity in 3/21 (14,28%) of the strains, and the other 3/21 have not secreted the enzyme. In 15/21 (71,41%), the activity was considered medium. Only 1/21 (4.76%) strain has not secreted phospholipase, other 2/21 (9.52%) have presented intermediary activity, and 19/21 (90,47%) were considered strong producers. The enzymatic activity of this enzyme showed difference between non-smokers and smokers ($P = 0,013$). For chondroitinase, 17/21 (80.95%) strains have presented high activity, 2/11 (9.52%) strains have presented intermediary activity, and 2/11 strains have not presented a

detectable activity. No strain has presented high activity of esterase/lipase in this group. The activity was intermediary in 19/21 (85.71%) of strains and absent in 3/21 (14.29%) of the isolates. In the production of haemolysin, only one strain has presented intermediary activity (4.76%) while 20/21 (95.23%) have presented high activity. The predominant zymotype was the Z22323.

The standard-strain CBS562 has presented a coinciding zymotype to the one found in the smokers, the Z23323, with production of all the researched enzymes, having elevated production in three of them (Graphic 3).

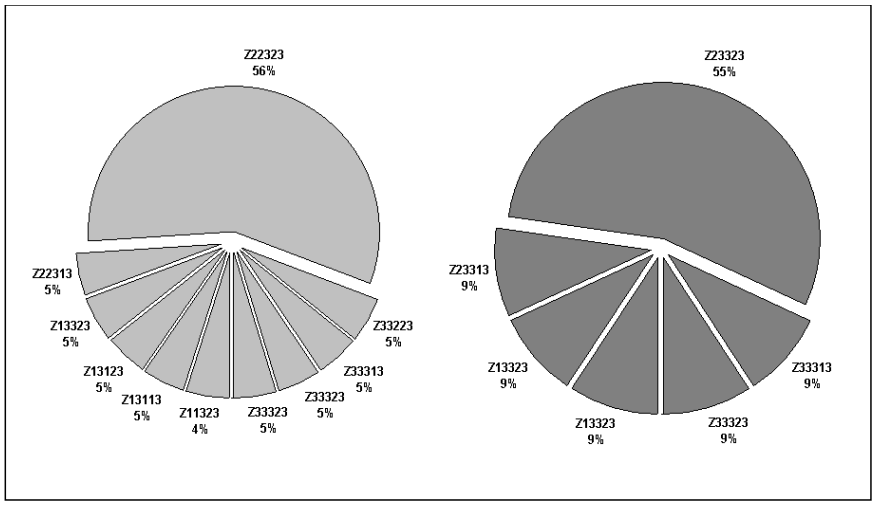


Fig 3. Distribution of zymotypes of *C. albicans* strains isolated from non-smokers (n = 11) and smokers (n = 21).

DISCUSSION

Despites the considerable source of information regarding the factors that determine the virulence of *C. albicans* (6, 13, 17, 35, 36), in our knowledge, no previous study has evaluated the enzymatic activity of the species, not even of the genus that was obtained from smoking and non-smoking individuals.

In the present study, 28,57% of smokers presented themselves positive for the colonization by *Candida* spp. against 39,65% of non-smokers ($P > 0.05$). These results

are less inferior to the ones published by Rassol *et al*. (29) that isolated yeasts in 43% of non-smokers and 57% of smokers. Nevertheless, not even these authors obtained statistic differences among groups, what would take us to an inference that the age might be a factor of predisposition to the more determinant colonization than the smoking habit.

C. albicans was the most prevalent species, being found in 78.54% of non-smokers and 72.41% of smokers, when colonized. Although there was found a higher percentage of isolation of this species, there is no statistical difference among the groups.

The production of secreted aspartyl-proteases (Saps) by the strains found in the present study corroborates with the studies of Bramono *et al*. (6), which found the activity of this enzyme in the strains of *C. albicans, C. tropicalis, C. parapsilosis, C. glabrata, C. krusei*, and *C. guilliermondii.* But, differently from the findings of Dóstal *et al.* (10), there was not found activity in strains of *C. glabrata*.

Regarding the phospholipases, there was not found any activity in the strains of *C. glabrata* and *C. parapsilosis*, what contradict the findings of other authors (11, 38), who affirm that these species are able to produce such enzymes. Such fact might have happened due to the reduced number of isolations of this species in the individuals who participated in this study.

When the secretion of these phospholipases is evaluated in isolates of *C. albicans* from non-smokers, it is noticeable a significant activity elevation (P = 0,013) in relation to the isolates of non-smokers. As it seems to be an enzyme directly related to the virulence of this species, it was expected higher secretion in the group of smokers. Such fact may be partially explained in function to random selection of the colonies that originated the analysed isolates. As there was selected only a colony of *C. albicans* per

individual, other strains with higher enzymatic activity might have been excluded from the study.

Ibrahim *et al.* (13) reported that commensal strains with low production of phospholipase are able to colonize mucosas, suggesting that the secretion of these enzymes is a prerequisite for the colonization of those type of epithelium, opinion have shared with Mayser *et al.* (21). As the individuals here analysed have not presented any clinic sign of oral candidosis, it is reasonable to suppose that this higher secretion of phospholipases by the strains of non-smokers might not have bigger implications in the simple event of colonization.

The totality of strains of *C. albicans* of the non-smoking group produced chondroitinase, in comparison to 90% produced by this species in the smoking group. Among the non-*albicans* only *C. tropicalis* and one strain of *C. parapsilosis* produced chondroitinase. More, the two strains of *C. glabrata* have not produced chondroitinase. This result corroborates with Shimizu *et al*. (39) who observed the production of the enzyme in 97.8% of the strains of *C. albicans*, being this production followed by *C. tropicalis, C. guilliermondii, C. parapsilosis*, and *C. krusei*; it also corroborates with Chaffin *et al.* (9) who affirmed that hyaluronidase and chondroitinase may be produced by *C. albicans, C. tropicalis, C. guilliermondii, C. parapsilosis,* and *C. krusei*, but in a small proportion of the isolates.

In the present study, there was evaluated also the secretion of esterase/lipase. All species were able to secrete this enzyme; however, in different proportions. This result corroborates with reported by another authors (6, 9, 33, 43) that this enzyme is present in a series of species of *Candida*; however, its correlation with the pathogenic behaviour of the strains was not established yet.

The activity of haemolysins is associated to the ability that these strains have for destroying red blood cells and use the iron as nutrient. Our results have shown that all species found have produced haemolysins, in concordance with the sstudy of Luo *et al.* (19), being strongly produced in the majority of strains.

Among the strains of examined *C. albicans*, none of them has presented high levels of secretion for the five enzymes, concomitantly, i.e. no one has presented a value 1-Pz higher than 0.640 for the five enzymes studied. This agrees with the study of Bramono *et al*. (6) who researched the secretion of secreted aspartyl-protease, lipase, and α-glucosidase in strains of *C. albicans*, and none of them has shown high levels of activity for those three enzymes, simultaneously.

The literature suggest that tobacco's cigarettes can cause changes in the oral epithelium that allowed the *Candida* colonization (3, 25). Willians *et al.* (44) showed that a higher keratinisation favours the adherence of *Candida albicans.* Moreover, Barret-Bee *et al*. (5) proposed that the phospholipase could have an important role in this adherence; a proposition not shared with Ghannoun *et al*. (12). In the same direction, Ray & Payne (30) showed that blastopores of *C. albicans* can degrade cellular keratin using acidic proteases and Jayatilake *et al*. (14) proposed that phospholipases can also be important in this destruction, what, without further appeal, would favour the colonization.

Concerning the arguments above, maybe, the lowest secretion of the enzymes per strains obtained from smokers is due to a clonal selection determined by the quality of the saliva. As the Saps (8) and the phospholipases (20, 34) are usually active only under acidic conditions and there was already shown that smokers present a more acidic saliva and smaller buffering ability than the non-smokers (17, 18, 27, 40), it is

reasonable to suppose that in this second group, it is not necessary a high rate of secretion for the strains to manage their colonization.

According with the available literature, smokers may show a higher oral Candidal colonization due to induction of increased epithelial keratinisation (4, 25, 44); to a reduction: in function of polymorphonuclear leukocytes, in gingival exudates and in salivary immunoglobulin A levels (42).

Researches of *Candida* species, especially *C. albicans*, his virulence and the effects of chemical substances about them, can facilitate the diagnosis, change the needs of treatment e help in the prevention of oral candidosis (6).

CONCLUSION

The results obtained in this study showed that in the two populations here analysed, the oral fungal load does not differ between smoker and non-smoker. Regarding the hydrolytic enzymes, there was a higher tendency of enzymatic secretion in the strains of *Candida albicans* isolated from non-smokers.

REFERENCES

1. **Almeida, O. P. and C. Scully.** 2002. Fungal infections of the mouth. Braz J Oral Sci. **1:** 19-26.
2. **Antley, P. P. and Hazen K. C.** 1988. Role of yeast cell growth temperature on *Candida albicans* virulence in mice. Infect. Immun. **56:** 2884-2890.
3. **Arendorf, T. M. and D. M Walker.** 1984. Tobacco smoking and denture wearing as local aetiological factors in median rhomboid glossitis. *Int J Oral Surg*. **13:** 411-415.
4. **Arrendondo, J., Nguyen V. T. and Chernyavsky A. I.** 2001. A Receptor-mediated mechanism of nicotine toxicity in oral candidal leukoplakia. Br. Dent. J. **155:** 340-343.
5. **Barrett-Bee K., Y. Hayes, R. G. Wilson and J. F. Ryley.** 1985. A comparison of phospholipase activity, cellular adherence and pathogenicity of yeasts. J Gen Microbiol. **131:** 1217-1221.
6. **Bramono K., M. Yamazaki, R. Tsuboi, H. Ogawa.** 2006. Comparison of proteinase, lipase and alpha-glucosidade activities from the clinical isolates of *Candida* species. Jpn. J. Infect. Dis. **59:** 73-76.
7. **Candido, R. C., C. Azevedo, R. V. P. Azevedo, M. C. Komesu.** 2000. Enzimotipagem de espécies do gênero *Candida* isoladas da cavidade bucal. Rev. Soc. Bras. Med. Trop. **33:** 437-442.
8. **Cassone A., F. De Bernardis, F. Mondello, T. Ceddia and L. Agatensi.** 1987. Evidence for a correlation between proteinase secretion and vulvovaginal candidosis. J. Infect. Dis. **156:** 777-783.

9. **Chaffin, W. L., J. L. López-Ribot, D. G. Casanova and J. P. Martinez.** 1998. Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. Microbiol. Mol. Biol. Rev. **62:** 130-180.
10. **Dostál, J., P. Hamal, L. Pavlíčková, M. Souček, T. Ruml, I. Pichová and O. Hrušková-Heidingsfeldová.** 2003. Simple method for screening *Candida* species isolates for the presence of secreted proteinases: a tool for the prediction of successful inhibitory treatment. J. Clin. Microbiol. **41:** 712-716.
11. **Ghannoum, M. A.** 2000. Potential role of phospholipases in virulence and fungal pathogenesis. Clin. Microbiol. Rev**. 13:** 122-143.
12. **Ghannoum M. A., S. G. Filler, A. S. Ibrahim, Y. Fu and J. E. Edwards Jr.** 1992. Modulation of interactions of *Candida albicans* and endothelial cells by fluconazole and amphotericin B. Antimicrob. Agents Chemother. **36:** 2239-2244.
13. **Ibrahim A. S., F. Mirbod, S. G. Filler, Y. Banno, G. T. Cole, Y. Kitajima, J. E. Edwards Jr., Y. Nozawa and M. A. Ghannoum.** 1995. Evidence implicating phospholipase as a virulence factor of *Candida albicans.* Infect. Immun. **63:** 1993-1998.
14. **Jayatilake J. A., Y. H. Samaranayake and L. P. Samaranayake.** 2005. An ultrastructural and a cytochemical study of candidal invasion of reconstituted human oral epithelium. J. Oral Pathol. Med. **34:** 240-246.
15. **Johnson, N.W. and C. A. Bain.** 2000. Tobacco and oral disease. British Dental Journal. **189:** 200-206.
16. **Kivela J., S. Parkkila, J. Metteri, A. K. Parkkila, A. Toivanen and H. Rajaniemi.** 1997. Salivary carbonic anhydrase VI concentration and its relation

to basic characteristics of saliva in young men. Acta Physiol. Scand. **161:** 221-225.

17. **Koga-Ito, C.Y., J. P. Lyon, V. Vidotto and M. A. de Resende.** 2006. Virulence factors and antifungal susceptibility of *Candida albicans* isolates from candidoses patients and control individuals. Mycopathologia. **161:** 219-223.
18. **Liede K. E., J. K. Haukka, J. H. Hietanen, M. H. Mattila, H. Ronka and T. Sorsa.** 1999. The association between smoking cessation and periodontal status and salivary proteinase levels. J. Periodontol. **70:** 1361-1368.
19. **Luo, G., L. P. Samaranayake and J. Y. Y. Yan.** 2001. *Candida* species exhibit differential in vitro hemolytics activities. J. Clin. Microbiol. **39:** 2971-2974.
20. **Lyon J. P. and M. A. de Resende.** 2006. Correlation between adhesion, enzyme production, and susceptibility to fluconazole in *Candida albicans* obtained from denture wearers. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. **102:** 632-638.
21. **Mayser, P., S. Laabs, K-U Heuer and K. Gründer.** 1996. Dection of extracelular phospholipase activity in *Candida albicans* and *Rhodotorula rubra.* Mycopathologia. **135:** 149-155.
22. **Menezes, E. A., M. S. Cavalcante, R. B. Farias, A. B. Teixeira, F. G. Pinheiro, B. P. Bezerra, J. C. N. Torres and F. A. Cunha.** 2005. Frequency and enzymatic activity of *Candida albicans* isolated from the buccal mucosa of children of a day-care center of the city hall of Fortaleza, Ceará, Brazil. J. Bras. Patol. Med. Lab. **41:** 9-13.
23. **Molero, G., R. Diez-Orejas,** F. **Navarro-Garcia,** L. **Monteoliva,** J. Pla, C. **Gil,** M. **Sanchez-Perez and** C. Nombela. 1998. *Candida albicans*: genetics, dimorphism and pathogenicity. Internatl. Microbiol. **1:** 95–106.

24. **Odds, F.C. and R. Bernaerts.** 1994. CHROMagar *Candida*, a new differential isolation medium for presumptive identification of clinically important *Candida* species. J. Clin. Microbiol. **32:** 1923-1929.

25. **Orellana-Bustos A.I., I. L. Espinosa-Santander, E. Franco-Martinez, N. Lobos-Jaimes-Freyre and A. V. Ortega-Pinto.** 2004. Evaluation of keratinization and AgNORs count in exfoliative cytology of normal oral mucosa from smokers and non-smokers. Med. Oral. **9:** 197-203.

26. **Ozkan, S., F. Kaynak, A. Kalkanci, U. Abbasoglu and S. Kustimur.** 2005. Slime production and proteinase activity of *Candida* species isolated from blood samples and the comparison of these activities with minimum inhibitory concentration values of antifungal agents. Mem. Inst. Oswaldo Cruz. **100:** 319-324.

27. **Parvinen, T.** 1984. Stimulated salivary flow rate, pH and lactobacillus and yeast concentrations in non-smokers and smokers. Scand. J. Dent. Res. **92:** 315-318.

28. **Price M. F., I. D. Wilkinson and L. O. Gentry.** 1982. Plate method for detection of phospholipase activity in *Candida albicans*. Sabouraudia. **20:** 7-14.

29. **Rassol, S., C. H. Siar and K. P. Ng.** 2005. Oral candidal species among smokers and non-smokers. JCPSP. **15:** 679-682.

30. **Ray, T.L. and C.D. Payne.** 1988. Scanning electron microscopy of epidermal adherence and cavitation in murine candidiasis: a role for *Candida* acid proteinase. Infect. Immun. **56:** 1942-1949.

31. **Rindum J.L., A. Stenderup and P. Holmstrup.** 1994. Identification of *Candida albicans* types related to healthy and pathological oral mucosa. J. Oral Pathol. Med. **23:** 406-412.

32. **Ruchel, J., R. Tegeler and M. A. Trost.** 1982. Comparison of secretory proteinases from different strains of *Candida albicans.* Sabouradia. **20:** 233-244.

33. **Rudek, W.** 1978. Esterase activity in *Candida* species. J. Clin. Microbiol. **8:** 756-759.

34. **Samaranayake L. P., J. M. Raeside and T. W. MacFarlane.** 1984. Factors affecting the phospholipase activity of *Candida* species in vitro. Sabouraudia. **22:** 201-207.

35. **Samaranayake, Y. H., R. S. Dassanayake, J. A. M. S. Jaytilake, B. P. K. Cheung, J. Y. Y. Yan, K. W. S. Yeung and L.P. Samaranayake**. 2005. Phospholipase B enzyme expression is not associated with other virulence attributes in *Candida albicans* isolates from patients with human immunodeficiency virus infection. J. Med. Microbiol. **54:** 583-593.

36. **Schaller, M., C. Borelli, H. C. Korting and B. Hube.** 2005. Hydrolytic enzymes as virulence factors of *Candida Albicans*. Mycoses. **48:** 365-377.

37. **Scully, C., M. El-Kabir and L. P. Samaranayake.** 1994. *Candida* and oral candidosis: a review. Crit. Rev. Oral Biol. Med. **5:** 125-157.

38. **Serda-Kantarcioğlu A. and A. Yücel.** 2002. Phospholipase and protease activity in clinical *Candida* isolates with reference to the sources of strains. Mycoses. **45:** 160-165.

39. **Shimizu M. T., A. O. Jorge, C. S. Unterkircher, V. Fantinato and C. R. Paula.** 1995. Hyaluronidase and chondroitin sulphatase production by different species of *Candida*. J. Med. Vet. Mycol. **33:** 27-31.

40. **Sitheeque M. A. M. and L. P. Samaranayake.** 2003. Chronic hyperplastic candidosis/candidiasis (candidal leukoplakia). Crit. Rev. Oral Biol. Med. **14:** 253-267.

41. **Smith R. F. and N. P. Willett.** 1968. Rapid plate method for screening hyaluronidase and chondroitin sulfatase producing microorganisms. Appl. Microbiol. **16:** 1434-1436.

42. **Soysa, N. S. and A. N. B. Ellepola.** 2005. The impact of cigarette/tobacco smoking on oral candidosis: an overview. Oral Diseases. **11:** 268-273.

43. **Tsuboi, R.**, **H. Komatsuzaki and H. Ogawa.** 1996. Induction of an extracellular esterase from *Candida albicans* and some of its properties. Infect. Immun. **64:** 2936-2940.

44. **Williams D. W., R. Walker, M. A. Lewis, R. T. Allison and A. J. Potts.** 1999. Adherence of *Candida albicans* to oral epithelial cells differentiated by Papanicolaou staining. J Clin Pathol. **52:** 529-531.

45. **Willis A. M., W. A. Coulter, C. R. Fulton, J. R. Hayes, P. M. Bell and P. J. Lamey.** 1999. Oral candidal carriage and infection in insulin-treated diabetic patients. Diabet Med. **16:** 675-679.

6. ANEXOS

ANEXO I – Material e Métodos

O projeto de pesquisa foi submetido e aprovado pelo Comitê de Ética em Pesquisa da PUCPR, sob registro no CEP nº 658/06 (Anexo V).

Amostra

Neste estudo foram avaliados 100 indivíduos, 42 não-fumantes e 58 fumantes. Foram considerados fumantes aqueles com consumo igual ou superior a cinco cigarros por dia há pelo menos um ano, e não-fumantes aqueles que relataram nunca terem fumado (29). Indivíduos que fumavam menos de cinco cigarros por dia foram excluídos da pesquisa. Cada indivíduo foi avaliado por um especialista em Estomatologia que realizou a anamnese e o exame físico intrabucal, inspecionando as mucosas, tecidos moles e dentes. Indivíduos com manifestações clínicas de candidose bucal; relato de doenças sistêmicas na anamnese, como diabetes ou hipertensão; mulheres grávidas; indivíduos que tivessem utilizado antibióticos nos três meses que precederam as coletas ou fazendo uso de medicação sialorredutora, foram excluídos da amostra. A média de idade dos indivíduos não-fumantes foi de 19,95 ± 2,13 anos e dos fumantes de 22,83 ± 5,75 anos. A média de cigarros consumidos por dia foi 12,79 ± 5,73 e do tempo de uso em anos foi de 6,19 ± 5,10. Amostras de raspado superficial (*swabbing*) do dorso da língua e da mucosa jugal dos indivíduos foram recolhidas, semeadas em placas contendo CHROMagar®*Candida* (CHROMagar Microbiology, Biomerieux, Paris, França) e incubadas a 37ºC, por 48 horas. Após a identificação fenotípica (24), as cepas foram transferidas para tubos de armazenamento contendo Ágar Sabouraud Dextrose (DAS, Difco Laboratories, Detroit, Mich., USA).

Ensaios enzimáticos

Um total de 43 cepas do gênero *Candida* (1 colônia/fenótipo por indivíduo) foram submetidas às provas de atividade enzimática. A cepa-padrão de *C. albicans* CBS562 proveniente do estoque do Laboratório de Estomatologia da PUCPR foi testada como controle. Os isolados foram crescidos em estufa a 37ºC em 5mL de Caldo Sabouraud Dextrose (Difco Laboratories, Detroit, Mich., USA). Após 24 horas as células foram recolhidas por centrifugação e lavadas três vezes com água destilada estéril. Os *pellets* foram ressuspendidos em água estéril até o equivalente ao tubo 10 da escala de MacFarland e 5 μL inoculados com o auxílio de discos de papel filtro esterilizados, em cinco pontos eqüidistantes, nos meios de cultura para detecção semi-quantitativa de aspartil-protease secretora (Sap), fosfolipases, condroitinase, esterase/lipase e hemolisinas. Todos os testes foram realizados em duplicata.

A secreção de Sap foi verificada seguindo o protocolo de Ruchel *et al.* (32). Uma suspensão contendo Ágar -15 g (Oxoid, Fakola AG, Basel, Suíça) e Yeast Nitrogen Base - 11,7 g (YNB, Difco Laboratories, Detroit, Mich., USA) e água destilada – 900 mL foi esterilizada por autoclave a 120ºC durante 15 minutos . Após resfriamento a 55ºC, esta suspensão foi acrescida de albumina bovina fração V - 2,0 g (Sigma Chem Co., St. Louis, Mo., USA), e 2,5 mL de Protovit Plus® (Roche, São Paulo, Brasil) dissolvidos em 100 mL de água destilada estéril e esterilizados em filtros de Millipore de 0,22 μm. O pH foi ajustado (entre 4,0 e 4,5), e a solução imediatamente vertida em placas de *petri* estéreis de 90 mm de diâmetro. As placas permaneceram em estufa a 37ºC por 10 dias (10).

A atividade fosfolipásica foi pesquisada seguindo o método de Price *et al.*(28). Em 1.000 mL de água destilada estéril foram dissolvidos 10,0g de peptona (Difco Laboratories, Detroit, Mich., USA), 30,0g de glucose, 57,3g de NaCl e 0,55g $CaCl_2$ e 20,0g de Ágar (Oxoid) . O meio foi esterelizado em autoclave a 120ºC por 15 minutos e

resfriado a uma temperatura de 55ºC. O *egg yolk* estéril sem telurito de potássio a 50% (Newprov Prod. Laborat., Inc., São José dos Pinhais, Paraná, Brasil) foi adicionado ao meio esterelizado. Após 4 dias de inoculação, as placas foram observadas quanto à formação de uma zona de cor amarelada ao redor das colônias,

Para a pesquisa de condroitinase foi empregado o protocolo de Smith & Willett (41) modificado. O meio de cultura foi preparado usando-se neopeptona 10g, glucose 40g, Ágar noble 15g (Sigma Chem Co., St. Louis, Mo., USA) e água destilada qsp 1.000mL e autoclavado a 120ºC por 15 minutos. Após resfriamento a 55ºC, foi acrescentada uma solução de albumina bovina fração V - 1,0g (Sigma) e sulfato de condroitina - 400mg dissolvidos em 10mL de água destilada estéril e esterilizados em filtros de *Millipore* de 0,22 μm. O pH final foi ajustado entre 6,8 ± 0,2. O halo formado foi medido após 48h de inoculação.

O teste de opacidade Tween® 80 (Sigma Chem Co., St. Louis, Mo., USA) foi utilizado para determinar a produção de esterase/lipase. O meio de cultura foi preparado com 15,0 g de SDA (Difco Laboratories, Detroit, Mich., USA) e 10 g $CaCl_2$, em 1.000 mL de água destilada (33). Após ser autoclavado, o meio foi resfriado a 50ºC e 10 mL de Tween®80 (Sigma) autoclavado foi adicionado. As inoculações permaneceram em estufa a 37ºC por sete dias consecutivos.

Para determinar a atividade hemolítica, as cepas foram semeadas em placas de Ágar-sangue usando o protocolo de Luo *et al.* Modificado (19), utilizando 70mL de sangue de ovelha fresco e desfibrinado (Newprov Prod. Laborat.); 30g de glucose; qsp 1.000mL de solução de Ágar-ágar 1% (Oxoid); pH final 5,6. A formação de um halo translúcido ao redor das colônias após 48h de incubação indicou atividade hemolítica positiva.

Obtenção dos resultados

Após os períodos de observação foram feitas as leituras do tamanho das colônias e dos halos formados por um único pesquisador calibrado, utilizando transiluminação e um paquímetro digital (Mitutoyo, 6" digital caliper, w/abs, Japão) e calculadas as atividades enzimáticas. Estas atividades foram determinadas de acordo com a técnica de Price *et al.* (28), dividindo-se o diâmetro da colônia (DC) pelo diâmetro do halo formado (DH). Este resultado é denominado Pz, que representa numericamente a atividade enzimática da cepa testada. Como menores valores Pz indicam maior atividade enzimática e vice-versa, optou-se por subtrair tais valores de um (1-Pz) (26). Os dados assim obtidos foram analisados quanto à sua normalidade de distribuição pelo teste de Kolmogorov-Smirnov. Para aspartil-protease secretora, condroitinase e hemolisinas houve distribuição normal dos valores e foi utilizado o teste *t* de Student. Nas enzimas onde não houve distribuição normal, fosfolipase e esterase/lipase, foi utilizado o teste não-paramétrico U de Mann-Whitney (Gráfico 2).

A atividade enzimática foi analisada estatisticamente somente nos isolados de *C. albicans*, devido à inexpressiva obtenção de outras espécies.

Além dos ensaios estatísticos, os valores 1-Pz também permitiram a classificação das cepas em zimotipos. Foram gerados dígitos a partir de um escore de atividade dado aos valores de Pz (7). Cepas não produtoras (Pz =1,000) foram codificadas como 1, pois o diâmetro do halo foi considerado igual ao da colônia; cepas com atividade média ($Pz \leq 0,639$) receberam código 2; cepas com atividade elevada ($0,999 \leq Pz \geq 0,640$) receberam código 3. Os zimotipos foram gerados seguindo a ordenação dos dígitos oriundos da secreção das enzimas aspartil-protease secretora, fosfolipases, condroitinase, esterase/lipase e hemolisinas. A zimotipagem nos forneceu uma visão geral da capacidade produtora de cada cepa, associando as cinco enzimas pesquisadas.

Questionário

Questionário Data: / / .

Voluntário nº:_____

Nome:_____

End.:_____

Aluno PUCPR?_____Curso:_____Período:_____

Data de nasc.:_____Idade:_____anos e_____meses

Há quanto tempo foi sua última refeição/ingestão de líquidos? _____

Fumante?_____Quantos cigarros por dia?_____

Qual marca?_____Fumante há quanto tempo?_____

Diabético?_____Compensado?_____

_

Hipertenso?_____Depressivo?_____

Toma algum medicamento?_____Qual (is)?_____

_____Há quanto tempo?_____

Boca seca?_____Quantos litros de líquido ingere por dia?_____

Transpiração noturna?_____

Usuário de prótese dentária?_____

Usuário de aparelho ortodôntico?_____

ANEXO II – Análise estatística

Indivíduos não-fumantes

Cepa	espécie	aspartil	fosfolipase	condroitinase	esterase	hemolisina
NF1	*C. albicans*	0,660	0,520	0,435	0,848	0,472
NF2	*C. albicans*	1,000	0,400	0,503	0,931	0,234
NF3	*C. albicans*	1,000	0,468	0,579	1,000	0,572
NF4	*C. albicans*	1,000	0,466	0,548	0,921	0,401
NF5	*C. albicans*	0,941	0,501	0,478	0,943	0,703
NF7	*C. albicans*	0,624	0,445	0,578	0,969	0,633
NF9	*C. albicans*	0,725	0,500	0,633	1,000	0,333
NF11	*C. albicans*	0,744	0,472	0,624	1,000	0,311
NF12	*C. albicans*	0,588	0,500	0,368	1,000	0,575
NF13	*C. albicans*	0,687	0,538	0,472	0,827	0,532
NF14	*C. albicans*	0,803	0,444	0,420	1,000	0,363
NF1d	*C. albicans*	0,788	0,456	0,510	1,000	0,548
NF2d	*C. albicans*	0,919	0,425	0,476	0,896	0,235
NF3d	*C. albicans*	1,000	0,534	0,518	0,944	0,419
NF4d	*C. albicans*	1,000	0,516	0,610	0,920	0,441
NF5d	*C. albicans*	0,957	0,454	0,429	1,000	0,460
NF7d	*C. albicans*	0,609	0,451	0,568	0,939	0,639
NF9d	*C. albicans*	0,766	0,529	0,625	0,715	0,357
NF11d	*C. albicans*	0,722	0,450	0,591	0,752	0,274
NF12d	*C. albicans*	0,576	0,474	0,378	1,000	0,657
NF13d	*C. albicans*	0,663	0,572	0,679	0,790	0,600
NF14d	*C. albicans*	0,751	0,571	0,385	1,000	0,238

Indivíduos fumantes

Cepa	espécie	aspartil	fosfolipase	condroitinase	esterase	hemolisina
GF1	*C. albicans*	0,799	0,464	0,338	0,794	0,356
GF2	*C. albicans*	0,575	0,425	0,432	1,000	0,325
GF3	*C. albicans*	0,637	0,580	0,449	0,794	0,347
GF5	*C. albicans*	0,891	0,462	0,538	0,828	0,439
GF6	*C. albicans*	0,865	0,814	0,440	1,000	0,519
GF7	*C. albicans*	0,741	0,500	0,471	1,000	0,397
GF8	*C. albicans*	0,430	0,457	0,649	0,855	0,508
GF9	*C. albicans*	0,627	0,476	0,429	0,748	0,239
GF10	*C. albicans*	1,000	0,791	0,531	0,633	0,360
GF12	*C. albicans*	0,632	0,615	0,654	0,967	0,344
GF14	*C. albicans*	1,000	0,516	0,407	0,796	0,307
GF15	*C. albicans*	0,838	0,475	0,476	0,855	0,513
GF16	*C. albicans*	0,814	0,478	0,666	0,938	0,412
GF17	*C. albicans*	1,000	0,473	0,626	0,835	0,506
GF18	*C. albicans*	0,802	0,492	0,395	1,000	0,494
GF19	*C. albicans*	1,000	0,491	1,000	1,000	0,500
GF20	*C. albicans*	0,883	0,531	0,391	0,763	0,251
GF21	*C. albicans*	0,794	0,477	0,610	0,954	0,672
GF22	*C. albicans*	1,000	0,586	1,000	0,934	0,326
GF23	*C. albicans*	0,851	0,500	0,792	1,000	0,487
GF27	*C. albicans*	1,000	1,000	0,353	0,941	0,484
GF1d	*C. albicans*	0,872	0,609	0,357	0,891	0,464
GF2d	*C. albicans*	0,688	0,588	0,633	1,000	0,296
GF3d	*C. albicans*	0,755	0,659	0,413	0,788	0,469
GF5d	*C. albicans*	0,797	0,562	0,695	0,873	0,444
GF6d	*C. albicans*	0,675	0,828	0,600	1,000	0,505
GF7d	*C. albicans*	0,789	0,500	0,692	0,747	0,327
GF8d	*C. albicans*	0,437	0,470	0,606	0,937	0,514
GF9d	*C. albicans*	0,599	0,450	0,500	1,000	0,321
GF10d	*C. albicans*	0,825	0,693	0,372	1,000	0,444
GF12d	*C. albicans*	0,639	0,533	0,679	0,790	0,321
GF14d	*C. albicans*	1,000	0,576	0,385	0,886	0,296
GF15d	*C. albicans*	0,788	0,450	0,556	1,000	0,497
GF16d	*C. albicans*	0,916	0,477	0,588	0,835	0,384
GF17d	*C. albicans*	0,817	0,501	0,652	0,952	0,488
GF18d	*C. albicans*	0,822	0,538	0,449	0,904	0,584
GF19d	*C. albicans*	1,000	0,476	1,000	1,000	0,586
GF20d	*C. albicans*	0,906	0,500	0,567	0,900	0,326
GF21d	*C. albicans*	0,672	0,490	0,641	0,944	0,612
GF22d	*C. albicans*	1,000	0,675	1,000	0,685	0,393
GF23d	*C. albicans*	0,902	0,471	0,750	0,858	0,542
GF27d	*C. albicans*	1,000	1,000	0,349	0,911	0,548

ANEXO II - Análise Estatística

Com a finalidade de se eliminar a hipótese nula de que as diferenças observadas entre os grupos são devidas ao acaso foi aplicado o teste de normalidade de Kolmogorov-Smirnov. Nas enzimas aspartil-protease, condroitinase e hemolisina, houve distribuição normal dos valores, portanto foi utilizado o teste "t" de Student. Nas enzimas onde não houve distribuição normal, fosfolipase e esterase/lipase, foi utilizado o teste não paramétrico U de Mann-Whitney. De acordo com este teste, para a atividade enzimática de fosfolipase, p=0,01, sendo estatisticamente significante, pois p< 0,05.

Testes de Normalidade		Kolmogorov-Smirnov		
	Grupos	Statistic	df	Sig.
condroitinase	Não Fumantes	0,114295346	22	0,2000
	Fumantes	0,113708348	42	0,1992
hemolisina	Não Fumantes	0,107259444	22	0,2000
	Fumantes	0,118425577	42	0,1514
fosfolipase	Não Fumantes	0,147174136	22	0,2000
	Fumantes	0,218649254	42	0,0000
esterase	Não Fumantes	0,205855777	22	0,0161
	Fumantes	0,145593267	42	0,0255
aspartil	Não Fumantes	0,151696582	22	0,2000
	Fumantes	0,130404093	42	0,0701

Grupos	**Variáveis**	**Count**	**Mean**	**Median**	**Std Deviation**
Não Fumantes	condroitinase	22	0,518	0,514	0,092
	hemolisina	22	0,454	0,450	0,149
	fosfolipase	22	0,486	0,473	0,046
	esterase	22	0,927	0,944	0,088
	aspartil	22	0,796	0,758	0,153
Fumantes	condroitinase	42	0,575	0,561	0,185
	hemolisina	42	0,432	0,444	0,104
	fosfolipase	42	0,563	0,500	0,140
	esterase	42	0,894	0,907	0,098
	aspartil	42	0,811	0,820	0,155

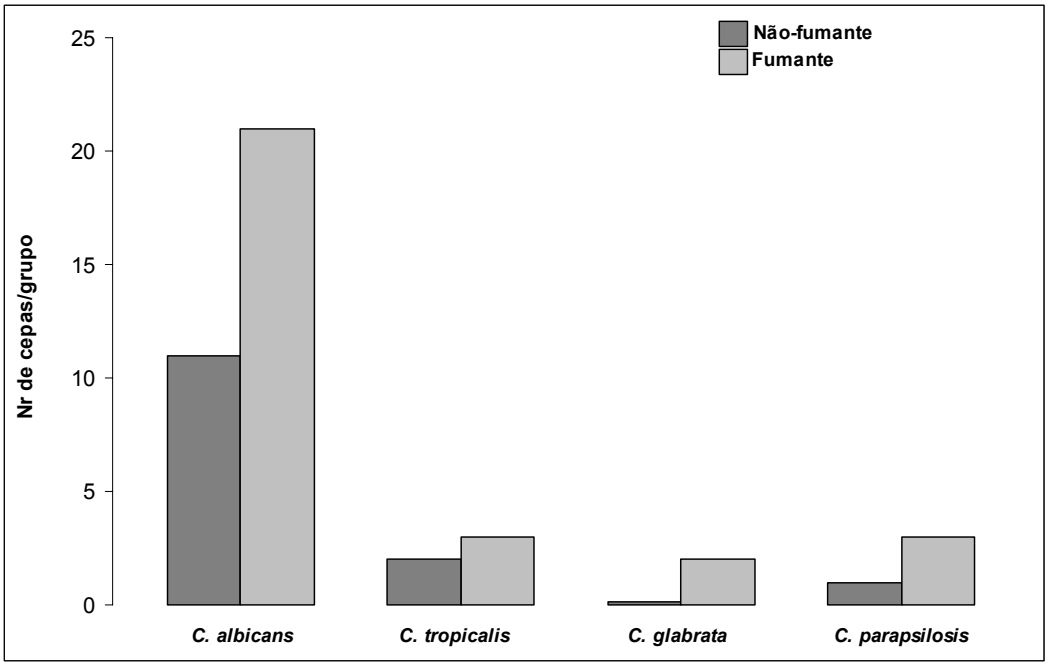
Teste U de Mann-Whitney

Ranks

	Grupos	N	Mean Rank	Sum of Ranks
fosfolipase	Não Fumantes	22	24,52272727	539,5
	Fumantes	42	36,67857143	1540,5
	Total	64		
esterase	Não Fumantes	22	36,59090909	805
	Fumantes	42	30,35714286	1275
	Total	64		

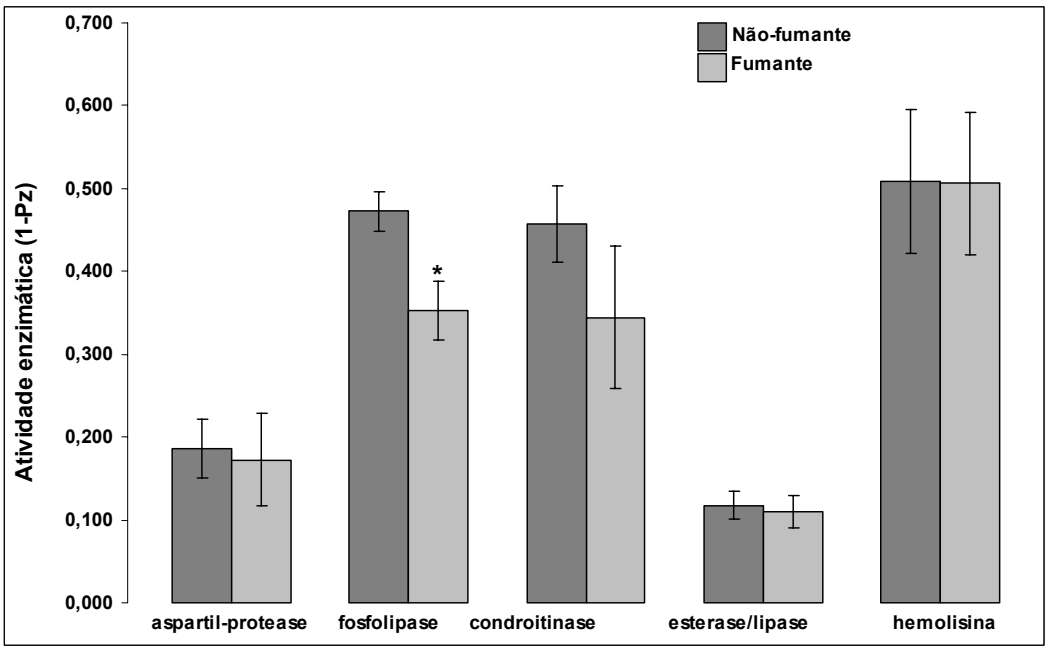
ANEXO III – GRÁFICOS

GRÁFICO 1. Distribuição das espécies isoladas



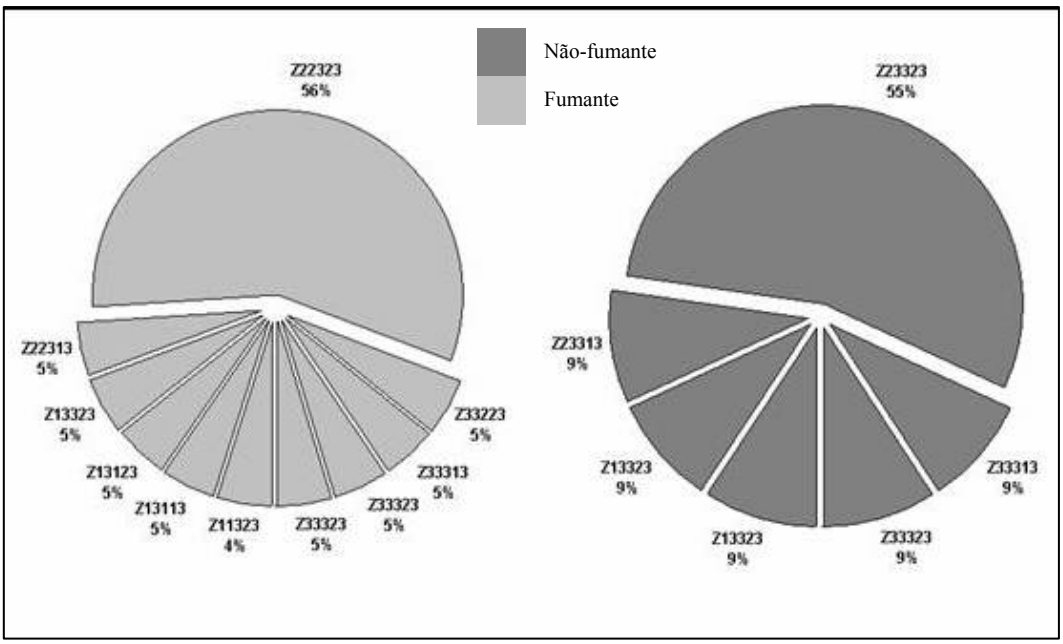
Graf. 1. Distribuição das espécies de *Candida* isoladas de não-fumantes (n=14) e fumantes.

GRÁFICO 2. Atividade enzimática das cepas de *C. albicans*



Graf. 2. Atividade das cepas de *C. albicans* isoladas de não-fumantes (n=11) e fumantes (n=21). Teste U de Mann-Whitney (p = 0.0131).

GRÁFICO 3. Distribuição dos zimotipos de *C. albicans*



Graf. 3. Distribuição dos zimotipos das cepas de *C. albicans* isoladas de não-fumantes (n=11) e fumantes (n=21).

ANEXO IV - Imagens

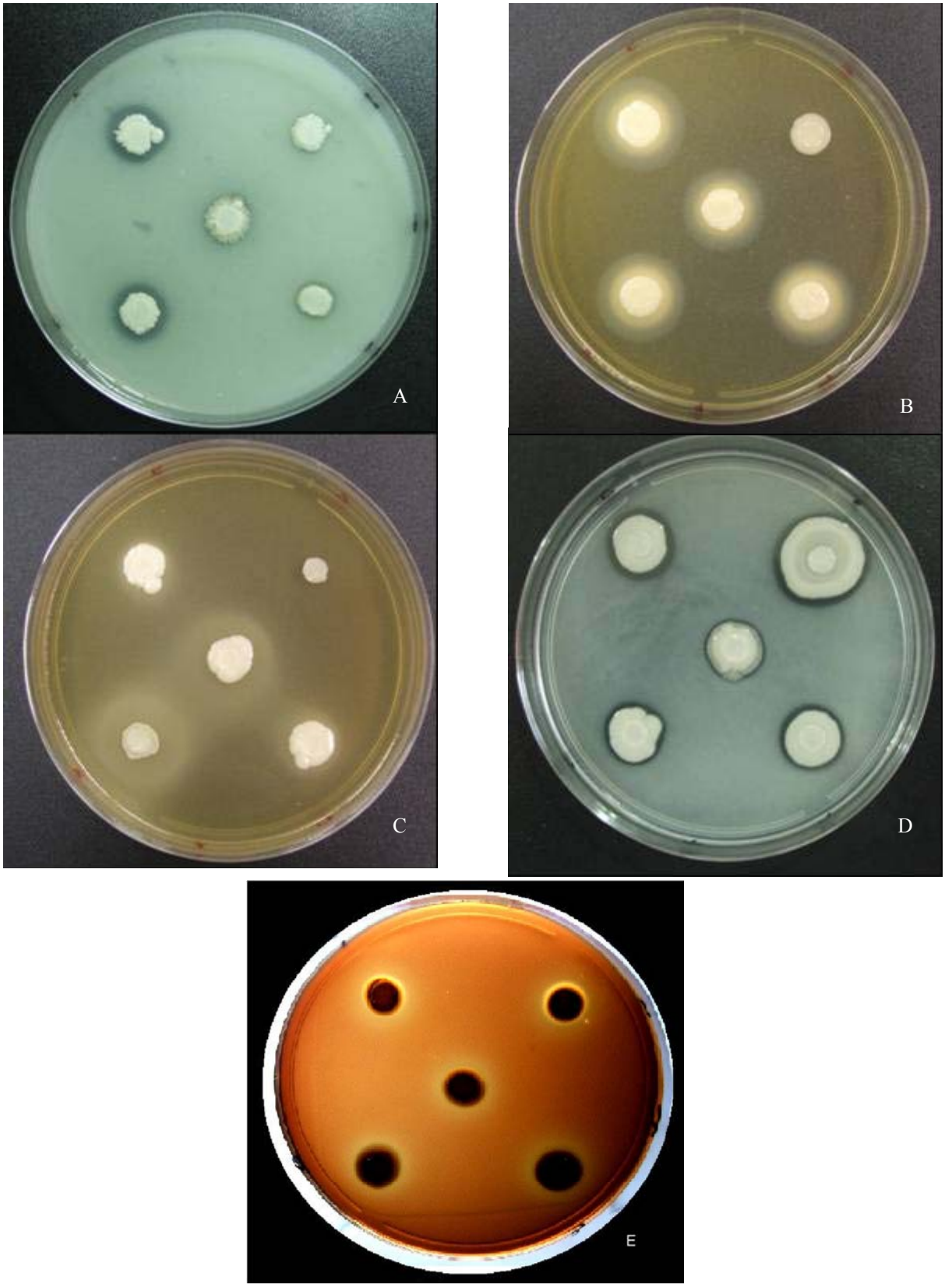


FIGURA 1. Halos de digestão característicos de cada enzima. A- aspartil-protease; B- fosfolipase; C- condroitinase; D- esterase/lipase e E- hemolisina.

ANEXO V – Termo de aprovação do Comitê de Ética em Pesquisa da PUCPR

Pontifícia Universidade Católica do Paraná
Pró-Reitoria Acadêmica e de Pesquisa
Núcleo de Bioética

Curitiba, 19 **de dezembro** de 2006.
Of. **658**/06/CEP-PUCPR
2ªvia solicitada

Ref. "**Diferenças nos perfis de virulência de cepas de cândida ssp. Isoladas de populações de tabagistas e não tabagistas**"

Prezado (a) Pesquisador (es),

Venho por meio deste informar a Vossa Senhoria que o Comitê de Ética em Pesquisa da PUCPR, no **dia 03 de agosto de 2005** aprovou o Projeto Intitulado "**Diferenças nos perfis de virulência de cepas de cândida ssp. Isoladas de populações de tabagistas e não tabagistas**", pertencente ao Grupo III, sob o registro no **CEP nº 658,** e será encaminhado a CONEP para o devido cadastro. Lembro ao senhor (a) pesquisador (a) que é obrigatório encaminhar relatório anual parcial e relatório final a este CEP.

Atenciosamente,

Profª M. Sc Ana Cristina Miguez Ribeiro
Coordenadora do Comitê de Ética em Pesquisa - PUCPR

Ilma Sra
Ana Claudia Santos de Azevedo Izidoro

Rua Imaculada Conceição, 1155 Prado Velho CEP 80215 901 Curitiba Paraná Brasil
Caixa Postal 17315 CEP 80242 980 Telefax: (41) 3271 2292 www.pucpr.br

ANEXO VI – Termo de Consentimento Livre e Esclarecido

TÍTULO DO PROJETO: DIFERENÇAS NOS PERFIS DE VIRULÊNCIA DE CEPAS DE *CANDIDA* spp. ISOLADAS DE POPULAÇÕES DE TABAGISTAS E NÃO-TABAGISTAS

Investigador: Cirurgiã Dentista Ana Claudia Santos de Azevedo Izidoro

Endereço: Pontifícia Universidade Católica do Paraná – Centro de Ciências Biológicas e da Saúde – Curso de Odontologia

Rua Imaculada Conceição 1155, CEP 80215-901, Curitiba – Paraná

Introdução

O pesquisador que conduz este projeto determinou que você atende aos requisitos iniciais para a participação no estudo. As informações a seguir descrevem o estudo no qual você terá o papel de participante. O pesquisador ou membro da equipe explicará os procedimentos e responderá a qualquer pergunta que você possa ter sobre este termo de consentimento informado e/ou sobre o estudo. Leia cuidadosamente este documento e não hesite em fazer perguntas sobre o objetivo do estudo ou sobre as informações fornecidas abaixo que você não compreenda. Nenhuma garantia pode ser feita quanto aos resultados do estudo.

Finalidade do estudo

A *Candida albicans* é um microorganismo que pode estar presente na mucosa bucal, na saliva ou ainda dentro de bolsas periodontais ao redor dos dentes de indivíduos com periodontite. Entretanto não se sabe ao certo a virulência desse microorganismo em pacientes fumantes e não-fumantes. O presente estudo tem por objetivo avaliar a produção de enzimas produzidas por este microorganismo e se microorganismos que estão presentes em pacientes fumantes têm a mesma virulência dos encontrados em pacientes não fumantes.

Descrição do estudo e procedimentos

Será preenchida uma ficha padronizada contendo dados pessoais, informações sobre o estado de saúde geral, tabagismo, tempo transcorrido após o último tratamento odontológico e uso de medicações.

Riscos e benefícios

De acordo com a metodologia exposta anteriormente acreditamos que o exame empregado neste estudo não seja capaz de produzir qualquer dano ou risco aos pacientes examinados. A coleta de saliva e de material na mucosa jugal não causa nenhum desconforto.

Receberei esclarecimentos sobre o que é candidose e como tratá-la e orientações sobre higiene bucal.

Perguntas relacionadas a este estudo

Você tem o direito de fazer perguntas com relação a este estudo a qualquer momento. Caso tenha novas perguntas, você poderá entrar em contato com Ana Claudia Santos de Azevedo Izidoro, no telefone 041-3274-5066.

Participação voluntária e direito de recusa/afastamento

Sua participação neste estudo é voluntária e não remunerada. Você poderá se recusar a participar ou poderá descontinuar sua participação a qualquer momento durante o estudo, sem penalidades ou perda de benefícios.

Confidencialidade

Os registros de sua participação neste estudo serão mantidos em confidência, de acordo com as leis municipais, estaduais e federais. O pesquisador e o Comitê de Ética em Pesquisa da Pontifícia Universidade Católica do Paraná (CEP-PUCPR) poderão inspecionar e ter acesso aos dados confidenciais que o identificam pelo nome. O CEP é um comitê que revisa os estudos para ajudar a assegurar que os direitos e o bem-estar dos pacientes e voluntários sejam protegidos e que o estudo seja conduzido eticamente. Qualquer publicação dos dados não o identificará. Assinando este formulário de consentimento, você autoriza o pesquisador a utilizar os dados, sem, contudo citar o seu nome.

Eu, _____, fui informado (a) dos objetivos específicos desta pesquisa, de forma clara e detalhada. Recebi informações específicas sobre cada procedimento no qual estarei envolvido, dos desconfortos, tanto quanto dos benefícios esperados. Todas as minhas dúvidas foram respondidas com clareza e sei que poderei solicitar novos esclarecimentos a qualquer momento. Além disso, sei que novas informações obtidas durante o estudo, me serão fornecidas e que terei liberdade de retirar meu consentimento de participação na pesquisa, se assim o desejar.

Fui informado que caso existam danos à minha saúde, causados diretamente pela pesquisa, terei direito a tratamento médico e indenização conforme estabelece a lei. Também sei que, caso existam gastos adicionais, estes serão absorvidos pelo orçamento da pesquisa. Autorizo a liberação dos registros médicos/odontológicos ao pesquisador e ao Comitê de Ética em Pesquisa da Pontifícia Universidade Católica do Paraná (CEP-PUCPR). Declaro ainda que recebí cópia do presente Termo de Consentimento

_____- _____ _____

Assinatura da paciente Nome (Local, Data e Hora)

_____- _____ _____

Assinatura do pesquisador Nome (Local, Data e Hora)

Este formulário foi lido para_____(nome do paciente) em ___/___/___ pelo_____ (nome do pesquisador) enquanto eu estava presente.

_____ - _____

Assinatura da testemunha Nome

ANEXO VII – Normas para publicação

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2006, p. 1–20 Vol. 72, No. 1
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.1.1–20.2006

APPLIED AND ENVIRONMENTAL MICROBIOLOGY

2006 INSTRUCTIONS TO AUTHORS*

SCOPE

Applied and Environmental Microbiology (AEM) publishes descriptions of all aspects of applied microbial research, basic research on microbial ecology, and research of a genetic and molecular nature that focuses on microbial topics of practical value. Research must address salient microbiological principles, fundamental microbial processes, or basic questions in applied or environmental microbiology. Topics that are considered include microbiology in relation to foods, agriculture, industry, biotechnology, public health, plants, and invertebrates and basic biological properties of bacteria, fungi, algae, protozoa, and other simple eukaryotic organisms as related to microbial ecology. Manuscripts should report new and significant findings that advance the understanding of microbiology and upon which other scientists may build.

The **microbial ecology** section covers a wide range of topics on the ecology of microorganisms, including culture-independent molecular assessments that provide new insights on (i) the structure-function relationships of microorganisms, (ii) the impact of in situ conditions on community structure, and (iii) the effect of changes in microbial community composition on ecosystem function. Archival phylogenetic snapshots that do not provide such insights are not acceptable for publication in AEM.

The **plant microbiology** section covers manuscripts dealing with all aspects of plant-microorganism interactions, including symbiotic and rhizosphere bacteria and phytopathogenic microorganisms.

New microbiological **methods** must provide novel avenues to address fundamental biological questions and will be considered for publication in AEM when accompanied by a demonstrated application. Descriptions of the application of previously described technologies, including the cloning, amplification, and expression of "foreign" genes, to a new genus or species of microbe will generally not be considered for independent publication. Manuscripts that describe the construction of engineered strains for innovative process application, development, or enhancement must present results to authenticate the utility, superiority, and uniqueness of such strains.

Manuscripts submitted to the **mycology** section should be clearly of a microbiological nature and may deal with basic biology, biochemistry, genetics, or physiology of fungi, molds, yeasts, or algae. Papers dealing purely with taxonomy or phylogeny, with fungal or algal structure, or with metabolism/alteration of metabolites/toxins by animal, plant, or insect cells, tissues, or organisms are not suitable. Documentation of the distribution/occurrence of toxins or metabolites in natural samples (foods, cereals, grains, soils, etc.) is suitable if the work includes studies involving the isolation, occurrence, or enumeration of the responsible microbes in these samples. The chemical or biochemical elucidation of metabolite or toxin structures is suitable if the work includes aspects of the enzymology or biosynthesis of these compounds.

Invertebrate microbiology manuscripts should address interactions between invertebrates and microorganisms, ranging from commensalism and mutualism to parasitism and pathogenicity. Manuscripts describing work dealing with the metabolites or toxins from animal, plant, or insect cells or the physiology of such cells are not suitable for AEM unless it affects a microbial community or individual microorganisms.

ASM publishes a number of different journals covering various aspects of the field of microbiology. Each journal has a prescribed scope which must be considered in determining the most appropriate journal for each manuscript. The following guidelines may be of assistance.

(i) AEM will consider manuscripts describing properties of enzymes and proteins that are produced by either wild-type or genetically engineered microorganisms and that are significant or have potential significance in industrial or environmental settings. Studies dealing with basic biological phenomena of enzymes or proteins or in which enzymes have been used in investigations of basic biological functions are more appropriate for the *Journal of Bacteriology*.

(ii) AEM will consider papers which describe the use of antimicrobial agents as tools for elucidating aspects of applied and environmental microbiology. Other papers dealing with antimicrobial agents, including manuscripts dealing with the biosynthesis and metabolism of such agents, are more appropriate for *Antimicrobial Agents and Chemotherapy*.

(iii) Papers on the biology of bacteriophages and other viruses are more appropriate for the *Journal of Virology* or the *Journal of Bacteriology*. AEM does, however, consider manuscripts dealing with viruses in relation to environmental, public health, or industrial microbiology.

(iv) Manuscripts dealing with the immune system or with topics of basic medical interest or oral microbiology are more appropriate for *Infection and Immunity*. Reports of clinical investigations and environmental biology applied to hospitals should be submitted to the *Journal of Clinical Microbiology*.

(v) AEM and *Eukaryotic Cell* (EC) accept manuscripts on population dynamics and the ecology of eukaryotic microbes. Studies of microbial communities and of microbial populations with identified economic or ecological significance, e.g., plant pathogens or symbionts, are usually more appropriate for AEM. Studies of single species of eukaryotes, especially "model" organisms or those without identified economic or ecological importance, are usually more appropriate for EC.

(vi) Manuscripts dealing with the purification and char-

* Shading indicates material that has been added or significantly updated.

acterization of enzymes or cloning of genes that have already been extensively described for other organisms will be considered for publication only if they offer experimentally supported new insights into the biological role, properties, or applications of these enzymes. Descriptions of genes or enzymes that differ only in minor ways from the prototypes are not suitable for AEM.

To best serve its readership, the journal must accept only those papers that are most significant to the field of applied and environmental microbiology. Thus, the editors will reject manuscripts that, while scientifically sound, represent only incremental extensions of other studies, are mainly confirmatory, or do not pursue a question in sufficient depth.

Questions about these guidelines may be directed to the editor in chief of the journal being considered.

If transfer to another ASM journal is recommended by an editor, the corresponding author will be contacted.

Note that a manuscript rejected by one ASM journal on scientific grounds or on the basis of its general suitability for publication is considered rejected by all other ASM journals.

EDITORIAL POLICY

Use of Microbiological Information

The Council Policy Committee (CPC) of the American Society for Microbiology affirms the long-standing position of the Society that microbiologists will work for the proper and beneficent application of science and will call to the attention of the public or the appropriate authorities misuses of microbiology or of information derived from microbiology. ASM members are obligated to discourage any use of microbiology contrary to the welfare of humankind, including the use of microbes as biological weapons. Bioterrorism violates the fundamental principles expressed in the Code of Ethics of the Society and is abhorrent to ASM and its members.

ASM recognizes that there are valid concerns regarding the publication of information in scientific journals that could be put to inappropriate use as described in the CPC resolution mentioned above. Members of the ASM Publications Board will evaluate the rare manuscript that might raise such issues during the review process. However, as indicated elsewhere in these Instructions, research articles must contain sufficient detail, and material/information must be made available, to permit the work to be repeated by others. Supply of materials should be in accordance with laws and regulations governing the shipment, transfer, possession, and use of biological materials and must be for legitimate, bona fide research needs. Links to, and information regarding, these laws and regulations can be found at http://www.asm.org/Policy/index.asp.

General Requirements

Manuscripts submitted to the journal must represent reports of original research, and the original data must be available for review by the editor if necessary.

All authors of a manuscript must have agreed to its submission and are responsible for its content (initial submission and any subsequent versions), including appropriate citations and acknowledgments, and must also have agreed that the corresponding author has the authority to act on their behalf in all matters pertaining to publication of the manuscript. The corresponding author is responsible for obtaining such agreements and for informing the coauthors of the manuscript's status throughout the submission, review, and publication process. For Authors' Corrections and Retractions, signed letters of agreement from all of the authors must be submitted (see p. 12).

By submission of a manuscript to the journal, **the authors guarantee that they have the authority to publish the work and that the manuscript, or one with substantially the same content, was not published previously, is not being considered or published elsewhere, and was not rejected on scientific grounds by another ASM journal.**

It is expected that the authors will provide written assurance that permission to cite unpublished data or personal communications has been granted.

By publishing in the journal, the authors agree that any DNAs, viruses, microbial strains, mutant animal strains, cell lines, antibodies, and similar materials newly described in the article are available from a national collection or will be made available in a timely fashion, at reasonable cost, and in limited quantities to members of the scientific community for noncommercial purposes. The authors guarantee that they have the authority to comply with this policy either directly or by means of material transfer agreements through the owner.

Similarly, the authors agree to make available computer programs, originating in the authors' laboratory, that are the only means of confirming the conclusions reported in the article but that are not available commercially. The program(s) and suitable documentation regarding its (their) use may be provided by any of the following means: (i) as a program transmitted via the Internet, (ii) as an Internet server-based tool, or (iii) as a compiled or assembled form on a suitable medium (e.g., magnetic or optical). It is expected that the material will be provided in a timely fashion and at reasonable cost to members of the scientific community for noncommercial purposes. The authors guarantee that they have the authority to comply with this policy either directly or by means of material transfer agreements through the owner.

Primary Publication

A scientific paper *or its substance* published in a serial, periodical, book, conference report, symposium proceeding, or technical bulletin, posted on a nonpersonal website, or made available through any other retrievable source, including CD-ROM and other electronic forms, is unacceptable for submission to an ASM journal on grounds of prior publication.

Posting of a method/protocol on a nonpersonal website should not interfere with the author's ability to have a manuscript utilizing that technique considered for publication in an ASM journal; however, ultimately, it is an editorial decision whether the method constitutes the substance of a paper.

Posting of a limited amount of original data on a personal/university/company website or websites of small collaborative groups working on a problem does not preclude subsequent submission to, and publication by, an ASM journal. The posted data, however, may not constitute the substance of the submission. Specific questions about this policy may be referred to the Publications Board chairman on a case-by-case basis. Posting of theses and dissertations on a personal/university-hosted website does not preclude subsequent submission to, and publication by, an ASM journal.

Posting of unpublished sequence data on the Internet is usually not considered prior publication; however, the address (URL) of the source of the sequence should be included in the text.

Preliminary disclosures of research findings webcast as meeting presentations or published in abstract form as adjuncts to a meeting, e.g., part of a program, are not considered prior publication.

It is incumbent upon the author to acknowledge any prior publication, including his own articles, of the data contained in a manuscript submitted to an ASM journal. A copy of the relevant work should be submitted with the paper as supporting material.

Ultimately, it is an editorial decision whether the material constitutes the substance of a paper.

Permissions

The corresponding author is responsible for obtaining permission from both the original author and the original publisher (i.e., the copyright owner) to reproduce or modify figures (including maps) and tables and to reproduce text (in whole or in part) from previous publications.

The original **signed** permission(s) must be submitted directly to the editor, outside the Rapid Review system, no later than the modification stage and should be identified as to the relevant item in the ASM manuscript (e.g., "permissions for Fig. 1 in AEM00123-06"). In addition, a statement indicating that the material is being reprinted with permission must be included in the relevant figure legend or table footnote of the manuscript. Reprinted text must be enclosed in quotation marks, and the permission statement must be included as running text or indicated parenthetically.

For supplemental material intended for posting by ASM (see p. 5), if the authors of the AEM manuscript are not also the owner of the supplemental material, the corresponding author **must** send to ASM **signed** permission from the copyright owner that allows posting of the material, as a supplement to the article, by ASM. The corresponding author is also responsible for incorporating in the supplemental material any copyright notices required by the owner.

Authorship

An author is one who made a substantial contribution to the overall design and execution of the experiments; therefore, **ASM considers all authors responsible for the entire paper.** Individuals who provided assistance, e.g., supplied strains or reagents or critiqued the paper, need not be listed as authors but may be recognized in the Acknowledgments section.

A study group, surveillance team, working group, consortium, or the like (e.g., the Active Bacterial Core Surveillance Team) may be listed as a coauthor in the byline if its contributing members satisfy the requirements for authorship and accountability as described in these Instructions. The names (and institutional affiliations if desired) of the contributing members only may be given in a footnote keyed to the study group name in the byline or as a separate paragraph in the Acknowledgments section.

If the contributing members of the group associated with the work do not fulfill the criteria of substantial contribution to and responsibility for the paper, the group may not be listed in the author byline. Instead, it and the names of its contributing members may be listed in the Acknowledgments section.

All authors must agree to the order in which their names are listed in the byline. Statements regarding equal contributions by two or more authors (e.g., X.J. and Y.S. contributed equally to . . .) are permitted as footnotes to bylines. Other statements of attribution may be included in the Acknowledgments section.

A change in authorship (order of listing or addition or deletion of a name) **after submission of the manuscript will be implemented only after receipt of signed statements of agreement from all parties involved.** Disputes about authorship may delay review and/or publication of the manuscript.

Conflict of Interest

All authors are expected to disclose, in the manuscript submittal letter, any commercial affiliations as well as consultancies, stock or equity interests, and patent-licensing arrangements that could be considered to pose a conflict of interest regarding the submitted article. (Inclusion of a company name in the author address lines of the manuscript does not constitute disclosure.) Details of the disclosure to the editor will remain confidential. However, it is the responsibility of authors to provide, in the Acknowledgments section, a *general* statement disclosing financial or other relationships that are relevant to the study. Examples of potentially conflicting interests that should be disclosed include relationships that might detract from an author's objectivity in presentation of study results, and interests whose value would be enhanced by the results presented. All funding sources for the project, institutional and corporate, should be cred-

ited in the Acknowledgments section, as described below. In addition, if a manuscript concerns a commercial product, the manufacturer's name must be indicated in the Materials and Methods section or elsewhere in the text, as appropriate, in an obvious manner.

Copyright

To maintain and protect the Society's ownership and rights and to continue to afford scientists the opportunity to publish in high-quality journals, ASM requires the corresponding author to sign a copyright transfer agreement on behalf of all the authors. This agreement is sent to the corresponding author when the manuscript is accepted and scheduled for publication. Unless this agreement is executed (*without changes and/or addenda*), ASM will not publish the manuscript.

In the copyright transfer agreement signed by an author, ASM grants to that author (and coauthors) the right to republish *discrete portions* of his (their) article in any other publication (print, CD-ROM, and other electronic forms) of which he is (they are) the author(s) or editor(s), *on the condition that appropriate credit is given to the original ASM publication.* This republication right also extends to posting on a host computer to which there is access via the Internet. Except as indicated below, significant portions of the article may *not* be reprinted/posted without ASM's prior written permission, however, as this would constitute duplicate publication.

Authors may post their own published articles on their *personal or university-hosted* (but not corporate, government, or similar) websites without ASM's prior written permission provided that appropriate credit is given (i.e., either the copyright lines shown on the top of the first page of the PDF version or "Copyright © American Society for Microbiology, [insert journal name, volume number, page numbers, and year]" for the HTML version).

The copyright transfer agreement asks that authors who were U.S. Government employees and who wrote the article as part of their employment duties be identified. This is because works authored solely by such U.S. Government employees are not subject to copyright protection, so there is no copyright to be transferred. The other provisions of the copyright transfer agreement, such as author representations of originality and authority to enter into the agreement, apply to U.S. Government employee-authors as well as to other authors.

Copyright for supplemental material (see p. 5) remains with the author, but a license permitting the posting by ASM will be sent, along with the article copyright transfer agreement, to the corresponding author for signing at the acceptance stage. (If the author of the article is not also the copyright owner of the supplemental material, the corresponding author **must** send to ASM **signed** permission from the owner that allows posting of the material, as a supplement to the article, by ASM. The corresponding author is also responsible for incorporating into the supplemental material any copyright notices required by the owner.)

ASM also requires that copyright transfer agreements be signed for cover artwork/photographs.

Funding Agency Repositories

The National Institutes of Health (NIH) requests that its grantee and intramural authors provide copies of their accepted manuscripts to PubMed Central (PMC) for posting in the PMC Public Access Repository. ASM allows such AEM authors to do so. ASM also allows AEM authors whose work was supported by similar funding agencies that have public access requirements like those of NIH (e.g., the Wellcome Trust) to post their **accepted manuscripts** in publicly accessible electronic repositories maintained by those funding agencies. If a funding agency does not itself maintain such a site, then ASM allows the author to fulfill that requirement by depositing the manuscript (**not** the typeset article) in an appropriate institutional or subject-based open repository established by a government or noncommercial entity.

Since ASM makes the final, typeset articles from its primary-research journals available free of charge on the ASM Journals and PMC websites 6 months after final publication, ASM recommends that when submitting the accepted manuscript to PMC or similar public access site, the author specify that the **posting release date for the manuscript be no earlier than 6 months after publication of the typeset article by ASM.**

Use of Human Subjects or Animals in Research

The use of human subjects or other animals for research purposes is regulated by the federal government and individual institutions. Manuscripts containing information related to human or animal use should clearly state that the research has complied with all relevant federal guidelines and institutional policies. Copies of these guidelines and policy statements must be available for review by the editor if necessary.

Nucleotide and Amino Acid Sequences

It is expected that newly determined nucleotide and/or amino acid sequence data will be deposited and GenBank/EMBL/DDBJ accession numbers will be included in the manuscript no later than the modification stage of the review process. It is also expected that the sequence data will be released to the public no later than the publication date of the article. The accession numbers should be included in a separate paragraph at the end of the Materials and Methods section for long-form papers or at the end of the text for short-form papers. If conclusions in a manuscript are based on the analysis of sequences and a GenBank/EMBL/DDBJ accession number is not provided at the time of the review, authors should provide the sequence data as supplemental material.

It is expected that, when previously published sequence accession numbers are cited in a manuscript,

the original citations (e.g., journal articles) will be included in the References section when possible or reasonable.

Authors are also expected to do elementary searches and comparisons of nucleotide and amino acid sequences against the sequences in standard databases (e.g., GenBank) immediately before manuscripts are submitted and again at the proof stage.

Analyses should specify the database, and the date of each analysis should be indicated in the format MM/YY. If relevant, the version of the software used should be specified.

See p. 14 for nucleic acid sequence formatting instructions.

The URLs of the databases mentioned above are as follows: DNA Data Bank of Japan (DDBJ), http://www.ddbj.nig.ac.jp; EMBL Nucleotide Sequence Submission (EMBL), http://www.ebi.ac.uk; and GenBank, National Center for Biotechnology Information (GenBank), http://www.ncbi.nlm.nih.gov.

Structural Determinations

It is expected that coordinates for new structures of macromolecules will be deposited in the Protein Data Bank and that assigned identification codes will be included in the manuscript no later than the modification stage of the review process. It is also expected that the coordinates will be released to the public no later than the publication date of the article. Authors are encouraged to send coordinates with their original submission, however, so that reviewers can examine them along with the manuscript. The accession number(s) should be listed in a separate paragraph at the end of the Materials and Methods section for full-length papers or at the end of the text for short-form papers.

The URLs for coordinate deposition are http://pdb.rutgers.edu and http://pdbdep.protein.osaka-u.ac.jp.

Microarray Data

It is expected that the entire set of supporting microarray data will be deposited in the appropriate public database (e.g., GEO, ArrayExpress, or CIBEX) and that the assigned accession number(s) will be included in the manuscript no later than the modification stage of the review process. It is also expected that the data will be released to the public no later than 6 months after publication of the typeset article. Authors are encouraged to send the relevant data with their original submission, however, so that reviewers can examine them along with the manuscript. The accession number(s) should be listed in a separate paragraph at the end of the Materials and Methods section for full-length papers or at the end of the text for short-form papers.

The URLs of the databases mentioned above are as follows: Gene Expression Omnibus (GEO), http://www.ncbi.nlm.nih.gov/geo; ArrayExpress, http://www.ebi.ac.uk/arrayexpress; and Center for Information Biology Gene Expression Database (CIBEX), http://cibex.nig.ac.jp.

Culture Deposition

AEM expects authors to deposit important strains in publicly accessible culture collections and to refer to the collections and strain numbers in the text. Since the authenticity of subcultures of culture collection specimens that are distributed by individuals cannot be ensured, authors should indicate laboratory strain designations and **donor sources** as well as original culture collection identification numbers.

Supplemental Material

Supplemental material intended for posting by ASM may not include additional figures or tables that simply support the authors' conclusions. It must be restricted to large or complex data sets or results that cannot be readily displayed in printed form because of space or technical limitations. Such material may include data from microarray, structural, biochemical, or video imaging analyses. In such cases, the manuscript submitted for review should include a distillation of the results so that the principal conclusions are fully supported without referral to the supplemental material.

Supplemental material intended for posting by ASM must be uploaded in Rapid Review and will be reviewed along with the manuscript. The decision to publish (i.e., post online only) the material with the article if it is accepted will be made by the editor and conveyed to the corresponding author in the acceptance e-mail. Note, therefore, it is possible that an article will be accepted but that the supplemental material will not be.

If the software required for users to view/use the supplemental material is not embedded in the file, you are urged to use shareware or generally available/easily accessible programs.

Unlike the article, supplemental material will **not** be edited by the ASM Journals staff and proofs will not be made available.

Supplemental material will always remain associated with its article and is **not** subject to any modifications after publication.

Material that has been published previously (print or online) is not acceptable for posting as supplemental data. Instead, the appropriate reference(s) to the original publication should be made in the article text.

Copyright for the supplemental material remains with the author, but a license permitting the posting by ASM will be sent, along with the article copyright transfer agreement, to the corresponding author for signing. If you are not the copyright owner, you **must** provide to ASM **signed** permission from the owner that allows posting of the material, as a supplement to your article, by ASM. You are responsible for including in the supplemental material any copyright notices required by the owner.

A one-time charge (amount not yet determined) may be levied for posting of supplemental material. When

instituted, the charge will be indicated in the ASM acceptance letter.

Compliance

Failure to comply with the policies described in these Instructions may result in a letter of reprimand, a suspension of publishing privileges in ASM journals, and/or notification of the authors' institutions.

Warranties and Exclusions

Articles published in this journal represent the opinions of the authors and do not necessarily represent the opinions of ASM. ASM does not warrant the fitness or suitability, for any purpose, of any methodology, kit, product, or device described or identified in an article. The use of trade names is for identification purposes only and does not constitute endorsement by ASM.

Page Charges

Authors whose research was supported by grants, special funds (including departmental and institutional), or contracts (including governmental) or whose research was done as part of their official duties (government, corporate, etc.) are required to pay page charges.

For a **corresponding author who is an ASM member,** page charges are currently $65 per page for the first six pages and $200 per page for each page in excess of six (subject to change without notice). To obtain the member rate, the *corresponding author* must be an ASM member.

For a **nonmember corresponding author,** page charges are currently $75 per page for the first six pages and $250 for each page in excess of six. A corresponding author who is not an ASM member may join ASM to obtain the member rate.

If the research was not supported by any of the means described above, a request to waive the charges may be mailed (Journals Department, ASM, 1752 N St., N.W., Washington, DC 20036-2904, USA) or faxed (202-942-9355) to the Journals Department. This request must indicate how the work was supported and should be accompanied by copies of the title page and Acknowledgments section.

Minireviews, Meeting Reviews, Guest Commentaries, and Comment Letters to the Editor are not subject to page charges.

Editorial Style

The editorial style of ASM journals conforms to the *ASM Style Manual for Journals* (American Society for Microbiology, 2006, in-house document) and *How To Write and Publish a Scientific Paper*, 5th ed. (Oryx Press, 1998), as interpreted and modified by the editors and the ASM Journals Department.

The editors and the Journals Department reserve the privilege of editing manuscripts to conform with the stylistic conventions set forth in the aforesaid publications and in these Instructions.

Review Process

All manuscripts are considered to be confidential and are reviewed by the editors, members of the editorial board, or qualified ad hoc reviewers. To expedite the review process, authors should recommend at least two reviewers who are not members of their institution(s) and have never been associated with them or their laboratory(ies); please provide their contact information where indicated on the submission form.

Copies of in-press and submitted manuscripts that are important for judgment of the present manuscript should be included as supporting material to facilitate the review.

When a manuscript is submitted to the journal, it is given a number (e.g., AEM00047-06 version 1) and assigned to one of the editors. (**Always refer to this number in communications with the editor and the Journals Department.**) *It is the responsibility of the corresponding author to inform the coauthors of the manuscript's status throughout the submission, review, and publication processes.* The reviewers operate under strict guidelines set forth in "Guidelines for Reviewers" (http://www.journals.asm.org/misc/reviewguide.shtml) and are expected to complete their reviews expeditiously.

The corresponding author is notified, generally within 4 to 6 weeks after submission, of the editor's decision to accept, reject, or require modification. When modification is requested, the corresponding author must either submit the modified version within 2 months or withdraw the manuscript. A point-by-point response to the reviews must be provided in the Rebuttal section of the Rapid Review submission form for the revised manuscript, and a compare copy of the manuscript (without figures) should be included as supporting material if the editor requested one.

Manuscripts that have been rejected, or withdrawn after being returned for modification, may be resubmitted if the major criticisms have been addressed. (**Note: A manuscript rejected by one ASM journal on scientific grounds or on the basis of its general suitability for publication is considered rejected by all other ASM journals.**) The cover letter must state that the manuscript is a resubmission, and the *former manuscript number should be provided* in the appropriate field on the submission form. **A point-by-point response** to the reviews and a compare copy of the revised manuscript showing the changes must be included as supporting material (the Rebuttal section appears in the submission form only if the manuscript is a modification). Resubmitted manuscripts are normally handled by the original editor.

Rejected manuscripts may be resubmitted only once unless permission has been obtained from the original editor or from the editor in chief.

Notification of Acceptance

When an editor has decided that a manuscript is acceptable for publication on the basis of scientific merit,

the author and the Journals Department are notified. The text files undergo an automated preediting, cleanup, and tagging process specific to the particular article type, and the illustrations are examined. If all files have been prepared according to the criteria set forth in these Instructions and those in Rapid Review, the acceptance procedure will be completed successfully. If there are problems that would cause extensive corrections to be made at the copyediting stage or if the files are not acceptable for production, ASM Journals staff will contact the corresponding author.

Once all the material intended for publication has been determined to be adequate, the manuscript is scheduled for the next available issue and an acceptance letter indicating the month of publication, approximate page proof dates, and table of contents section is mailed to the corresponding author; a copyright transfer agreement is also included, as is a license to permit posting of supplemental material (if applicable). The editorial staff of the ASM Journals Department completes the editing of the manuscript to bring it into conformity with prescribed standards.

Page Proofs

Page proofs, together with a query sheet and instructions for handling proofs, will be made available to the corresponding author electronically via a PDF file that can be accessed through a unique password. Since corresponding authors will be notified of the availability of their PDF proofs, instructed how to access information about page charges, reprints, and color figure charges (if applicable), and assigned their unique password via e-mail, an e-mail address **must** be supplied in the correspondent footnote. Failure to do so may result in a delay in publication. **The PDF page proofs must be printed out, and corrections must be written on the hard copy.** Queries must be answered on the query page or on a separate sheet of paper, and any changes related to the queries must be indicated on the proofs. Note that the copy editor does not query at every instance where a change has been made. Queries are written only to request necessary information or clarification of an unclear passage or to draw attention to edits that may have altered the sense. It is the author's responsibility to read the entire text, tables, and figure legends, not just items queried. As soon as the page proofs are corrected and signed by the person who proofread them (within 48 h), **they should be mailed or sent by a courier service** such as FedEx, **not** faxed or sent as an e-mail attachment, to the ASM Journals Department.

The proof stage is not the time to make extensive corrections, additions, or deletions. Important new information that has become available between acceptance of the manuscript and receipt of the proofs may be inserted as an addendum in proof with the permission of the editor. If references to unpublished data or personal communications are added, it is expected that written assurance granting permission for the citation will be included. Limit changes to correction of spelling errors, incorrect data, and grammatical errors and updated information for references to articles that have been submitted or are in press. If URLs have been provided in the article, recheck the sites to ensure that the addresses are still accurate and the material that you expect the reader to find is indeed there.

Questions about *late proofs and problems in the proofs* should be directed to the ASM Journals Department (telephone, 202-942-9219). Questions about *accessing or viewing your PDF proofs* should be directed to Katie Gay of Cadmus Professional Communications at 804-261-3155 or gayk@cadmus.com.

Reprints

Reprints (in multiples of 100) may be purchased by all coauthors. In the proof notification e-mail, the corresponding author will be instructed how to access information about reprints.

The corresponding authors of Minireviews, Meeting Reviews, and Guest Commentaries will receive 100 free reprints of their contribution; additional reprints (in multiples of 100) may be purchased if desired. As for regular articles, the corresponding author will be instructed, in the proof notification e-mail, how to access information about reprints.

PDF Files

A corresponding author who has included an e-mail address in his "corresponding author" footnote will have limited access (10 downloads, total) to the PDF file of his published article. An e-mail alert will automatically be sent to him on the day the issue is posted. It will provide a URL, which will be required to obtain access, and instructions. An article may be viewed, printed, or stored, provided that it is for the author's own use.

Should coauthors or colleagues be interested in viewing the paper for their own use, the corresponding author may provide them with the URL; a copy of the article may not be forwarded electronically. However, they must be made aware of the terms and conditions of the ASM copyright. (For details, go to http://www.journals.asm.org/misc/terms.shtml.) Note that each such download will count toward the corresponding author's total of 10. After 10 downloads, access will be denied and can be obtained only through a subscription to the journal (either individual or institutional) or after the standard access control has been lifted (i.e., 6 months after publication).

HOW TO SUBMIT MANUSCRIPTS

All submissions to AEM must be made electronically via the Rapid Review online submission and peer review system at the following URL: www.rapidreview.com/ASM2/author.html. (E-mailed submissions will not be accepted.) First-time users must create an Author ac-

count, which may be used for submitting to all ASM journals. Instructions for creating an Author account are available at the above URL under the Create Account button. The person from whose account a submission is made will be recorded as the corresponding author, and any correspondence will be sent to the e-mail address in that account. Step-by-step instructions for submitting a manuscript via Rapid Review are available from the account holder's My Manuscripts page. Information on file types acceptable for electronic submission can be found under the More About File Formats button.

ORGANIZATION AND FORMAT

On receipt at ASM, an accepted manuscript undergoes an automated preediting, cleanup, and tagging process specific to the particular article type. To optimize this process, manuscripts must be supplied in the correct format and with the appropriate sections and headings.

Type every portion of the manuscript double spaced (a minimum of 6 mm between lines), including figure legends, table footnotes, and References, and number all pages in sequence, including the abstract, figure legends, and tables. Place the last two items after the References section. **Manuscript pages should have line numbers; manuscripts without line numbers may be editorially rejected by the editor, with a suggestion of resubmission after line numbers are added.** The font size should be no smaller than 12 points. It is recommended that the following sets of characters be easily distinguishable in the manuscript: the numeral zero (0) and the letter "oh" (O); the numeral one (1), the letter "el" (l), and the letter "eye" (I); and a multiplication sign (×) and the letter "ex" (x). Do not create symbols as graphics or use special fonts that are external to your word processing program; use the "insert symbol" function. Set the page size to 8½ by 11 inches (ca. 21.6 by 28 cm). Italicize or underline any words that should appear in italics, and indicate paragraph lead-ins in bold type.

Authors who are unsure of proper English usage should have their manuscripts checked by someone proficient in the English language.

Manuscripts may be editorially rejected, without review, on the basis of poor English or lack of conformity to the standards set forth in these Instructions.

Manuscript Submission Checklist:

- Double space all text, including references and figure legends
- Number pages
- Number lines
- Present statistical treatment of data where appropriate
- Format references in ASM style
- Indicate journal section for manuscript publication
- Provide accession numbers for all sequences or sequence alignments important for evaluation of the manuscript as supplemental material or make the material available on a website for access by the editor and reviewers
- Confirm that genetic and chemical nomenclature conforms to instructions
- Include as supporting material in-press and submitted manuscripts that are important for judgment of the present manuscript

Long-Form Papers

Long-form papers should include the elements described in this section.

Title, running title, and byline. Each manuscript should present the results of an independent, cohesive study; thus, numbered series titles are not permitted. Exercise care in composing a main title. Avoid the main title/subtitle arrangement, complete sentences, and unnecessary articles. On the title page, include the title, running title (not to exceed 54 characters and spaces), name of each author, address(es) of the institution(s) at which the work was performed, each author's affiliation, and a footnote indicating the present address of any author no longer at the institution where the work was performed. Place an asterisk after the name of the author to whom inquiries regarding the paper should be directed (see "Correspondent footnote" below).

Study group in byline. A study group, surveillance team, working group, consortium, or the like (e.g., the Active Bacterial Core Surveillance Team) may be listed as a coauthor in the byline if its contributing members satisfy the requirements for authorship and accountability as described in these Instructions. The names (and institutional affiliations if desired) of the contributing members may be given in a footnote keyed to the study group name in the byline or as a separate paragraph in Acknowledgments.

If the contributing members of the group associated with the work do not fulfill the criteria of substantial contribution to and responsibility for the paper, the group may not be listed in the author byline. Instead, it and the names of its contributing members may be listed in the Acknowledgments section.

Correspondent footnote. The complete mailing address, a single telephone number, a single fax number, and a single e-mail address for the corresponding author should be included on the title page of the manuscript. This information will be published in the article as a footnote to facilitate communication, and the e-mail address will be used to notify the corresponding author of availability of proofs and, later, of the PDF file of the published article.

Abstract. Limit the abstract to **250 words or fewer** and concisely summarize the basic content of the paper with-

out presenting extensive experimental details. Avoid abbreviations and references, and do not include diagrams. When it is essential to include a reference, use the same format as shown for the References section but omit the article title. Because the abstract will be published separately by abstracting services, it must be complete and understandable without reference to the text.

Introduction. The introduction should supply sufficient background information to allow the reader to understand and evaluate the results of the present study without referring to previous publications on the topic. The introduction should also provide the hypothesis that was addressed or the rationale for the present study. Use only those references required to provide the most salient background rather than an exhaustive review of the topic.

Materials and Methods. The Materials and Methods section should include sufficient technical information to allow the experiments to be repeated. When centrifugation conditions are critical, give enough information to enable another investigator to repeat the procedure: make of centrifuge, model of rotor, temperature, time at maximum speed, and centrifugal force ($\times g$ rather than revolutions per minute). For commonly used materials and methods (e.g., media and protein concentration determinations), a simple reference is sufficient. If several alternative methods are commonly used, it is helpful to identify the method briefly as well as to cite the reference. For example, it is preferable to state "cells were broken by ultrasonic treatment as previously described (9)" rather than to state "cells were broken as previously described (9)." The reader should be allowed to assess the method without constant reference to previous publications. Describe new methods completely, and give sources of unusual chemicals, equipment, or microbial strains. When large numbers of microbial strains or mutants are used in a study, include tables identifying the immediate sources (i.e., sources from whom the strains were obtained) and properties of the strains, mutants, bacteriophages, plasmids, etc.

A method, strain, etc., used in only one of several experiments reported in the paper may be described in the Results section or very briefly (one or two sentences) in a table footnote or figure legend. It is expected that the sources from whom the strains were obtained will be identified.

Results. In the Results section, include only the results of the experiments; reserve extensive interpretation of the results for the Discussion section. Present the results as concisely as possible in **one** of the following: text, table(s), or figure(s). Avoid extensive use of graphs to present data that might be more concisely presented in the text or tables. For example, except in unusual cases, double-reciprocal plots used to determine apparent K_m values should not be presented as graphs; instead, the values should be stated in the text. Similarly, graphs illustrating other methods commonly used to derive kinetic or physical constants (e.g., reduced-viscosity plots and plots used to determine sedimentation velocity) need not be shown except in unusual circumstances. Limit photographs (particularly photomicrographs and electron micrographs) to those that are absolutely necessary to show the experimental findings. Number figures and tables in the order in which they are cited in the text, and be sure to cite all figures and tables.

Discussion. The Discussion should provide an interpretation of the results in relation to previously published work and to the experimental system at hand and should not contain extensive repetition of the Results section or reiteration of the introduction. In short papers, the Results and Discussion sections may be combined.

Acknowledgments. The source of any financial support received for the work being published must be indicated in the Acknowledgments section. (It will be assumed that the absence of such an acknowledgment is a statement by the authors that no support was received.) The usual format is as follows: "This work was supported by Public Health Service grant CA-01234 from the National Cancer Institute."

Recognition of personal assistance should be given as a separate paragraph, as should any statements disclaiming endorsement or approval of the views reflected in the paper or of a product mentioned therein.

Appendixes. Appendixes, which contain additional material to aid the reader, are permitted. Titles, authors, and References sections that are distinct from those of the primary article are not allowed. If it is not feasible to list the author(s) of the appendix in the byline or the Acknowledgments section of the primary article, rewrite the appendix so that it can be considered for publication as an independent article, either long-form or short-form style. Equations, tables, and figures should be labeled with the letter "A" preceding the numeral to distinguish them from those cited in the main body of the text.

References. (i) Works listed in References. The References section must include all journal articles (both print and online), books and book chapters (both print and online), patents, theses and dissertations, and published conference proceedings (not abstracts; see below), as well as in-press journal articles, book chapters, and books (publication title must be given). Arrange the citations in **alphabetical order** (letter by letter, ignoring spaces and punctuation) by first author and **number consecutively.** Provide the names of **all** the authors for each reference. All listed references **must** be cited parenthetically by number in the text. Since title and byline information that is downloaded from PubMed does not show accents, italics, or special characters, authors should refer to the PDF files or hard-copy versions of

the articles and incorporate the necessary corrections in the submitted manuscript. Abbreviate journal names according to *BIOSIS Serial Sources* (BIOSIS, Philadelphia, Pa., 2005).

Follow the styles shown in the examples below.

Print references:

1. **Arendsen, A. F., M. Q. Solimar, and S. W. Ragsdale.** 1999. Nitrate-dependent regulation of acetate biosynthesis and nitrate respiration by *Clostridium thermoaceticum*. J. Bacteriol. **181**:1489–1495.
2. **Cox, C. S., B. R. Brown, and J. C. Smith.** J. Gen. Genet., in press.[¶] {*Article title is optional; journal title is mandatory.*}
3. **da Costa, M. S., M. F. Nobre, and F. A. Rainey.** 2001. Genus I. *Thermus* Brock and Freeze 1969, 295,[AL] emend. Nobre, Trüper and da Costa 1996b, 605, p. 404–414. *In* D. R. Boone, R. W. Castenholz, and G. M. Garrity (ed.), Bergey's manual of systematic bacteriology, 2nd ed., vol. 1. Springer, New York, N.Y.
4. **Elder, B. L., and S. E. Sharp.** 2003. Cumitech 39, Competency assessment in the clinical laboratory. Coordinating ed., S. E. Sharp. ASM Press, Washington, D.C.
5. **Fitzgerald, G., and D. Shaw.** *In* A. E. Waters (ed.), Clinical microbiology, in press. EFH Publishing Co., Boston, Mass.[¶] {*Chapter title is optional.*}
6. **Forman, M. S., and A. Valsamakis.** 2003. Specimen collection, transport, and processing: virology, p. 1227–1241. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, J. H. Jorgensen, and R. H. Yolken (ed.), Manual of clinical microbiology, 8th ed. ASM Press, Washington, D.C.
7. **Green, P. N., D. Hood, and C. S. Dow.** 1984. Taxonomic status of some methylotrophic bacteria, p. 251–254. *In* R. L. Crawford and R. S. Hanson (ed.), Microbial growth on C_1 compounds. Proceedings of the 4th International Symposium. American Society for Microbiology, Washington, D.C.
8. **Odell, J. C.** April 1970. Process for batch culturing. U.S. patent 484,363,770. {*Include the name of the patented item/process if possible.*}
9. **O'Malley, D. R.** 1998. Ph.D. thesis. University of California, Los Angeles. {*Title is optional.*}

[¶]A reference to an in-press ASM publication should state the control number (e.g., AEM00577-06) if it is a journal article or the name of the publication if it is a book.

Online references:

1. **Charlier, D., and N. Glansdorff.** September 2004, posting date. Biosynthesis of arginine and polyamines. *In* R. Curtiss III et al. (ed.), EcoSal—*Escherichia coli* and *Salmonella*: cellular and molecular biology, chapter 3.6.1.10. [Online.] http://www.ecosal.org. ASM Press, Washington, D.C. {*For online-only books or continually updated Web resources [for the latter, posting or accession date required, but publisher's name and location optional].*}
2. **Dimick, J. B., H. G. Welch, and J. D. Birkmeyer.** 18 August 2004, posting {*or* revision} date. Surgical mortality as an indicator of hospital quality. JAMA **292**. [Online.] http://jama.ama-assn.org/cgi/content/short/292/7/847. {*For online journals; page numbers may not be available.*}
3. **Sullivan, C. J. (ed.).** 1999–2001. Fungi: an evolving electronic resource for the microbiological community. ASM Press. [Online.] http://link.asmusa.de/link/service/books/91090. Accessed 7 September 2001. {*For online-only books.*}
4. **Zellnitz, F., and P. M. Foley.** 2 October 1998, posting {*or* revision} date. History of virology. Am. Virol. J. **1**:30–50. [Online.] http://www.avj.html. {*For online-only journals; page numbers may not be available.*}
5. **Zheng, Z., and J. Zou.** 5 September 2001. The initial step of the glycerolipid pathway: identification of glycerol-3-phosphate/dihydroxyacetone phosphate dual substrate acyltransferases in Saccharomyces cerevisiae. J. Biol. Chem. doi:10.1074/jbc.M104749200. {*For papers published online in manuscript form.*}

NOTE: A URL or DOI is necessary for each online-only reference; a posting or accession date is required for any online reference that is periodically updated or changed.

(ii) Items cited in the text. References to unpublished data, articles submitted for publication, meeting abstracts (including those published in journal supplements), personal communications, letters (irrespective of type) and authors' replies to letters, company publications, patent applications and patents pending, computer software, databases, and websites should be made parenthetically in the text as follows.

. . . similar results (R. B. Layton and C. C. Weathers, unpublished data).

. . . system was used (J. L. McInerney, A. F. Holden, and P. N. Brighton, submitted for publication).

. . . in mitochondria (S. De Wit, C. Thioux, and N. Clumeck, Abstr. 34th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 114, 1994).

. . . for other bacteria (A. X. Jones, personal communication).

. . . discussed previously (L. B. Jensen, A. M. Hammerum, R. L. Poulsen, and H. Westh, Letter, Antimicrob. Agents Chemother. **43**:724–725, 1999).

. . . discussed previously (S. L. W. On and P. A. R. Vandamme, Authors' Reply to Letter, J. Clin. Microbiol. **39**:2751–2752, 2001).

. . . the manufacturer (Sigma manual, Sigma Chemical Co., St. Louis, Mo.).

. . . this process (V. R. Smoll, 20 June 1999, Australian Patent Office). {*For non-U.S. patent applications, give the date of publication of the application.*}

. . . information found at the XYZ website (http://cbx_iou.pgr).

. . . the ABC program (version 2.2; Department of Microbiology, State University [http://www.stu.micro]).

URLs for companies that produce any of the products mentioned in your study or for products being sold may NOT be included in the article. However, company URLs that permit access to scientific data related to the study or to shareware used in the study are permitted.

Short-Form Papers

The short-form format is intended for the presentation of brief observations that do not warrant full-length papers. Submit short-form papers in the same way as full-length papers. *They receive the same review, they are not published more rapidly than full-length papers, and they are not considered preliminary communications.*

The title, running title (not to exceed 54 characters and spaces), byline, and correspondent footnote should be prepared as for the long-form paper. Each short-form paper must have an **abstract of no more than 50 words.** Do not use section headings in the body of the paper; combine methods, results, and discussion in a single section. Paragraph lead-ins are permissible. The text should be kept to a minimum and, if possible, **should not exceed 1,000 words**; the number of figures and tables should also be kept to a minimum. **Materials and methods should be described in the text, not in figure legends or table footnotes.** Present acknowledgments as in long-form papers, but do not use a heading. The References section is identical to that of long-form papers.

Minireviews

Minireviews are brief (**limit of 6 printed pages exclusive of references**) biographical profiles, historical perspectives, or summaries of developments in fast-moving areas. They must be based on published articles; they may address any subject within the scope of AEM.

Minireviews may be either solicited or proffered by authors responding to a recognized need. Irrespective of origin, Minireviews are subject to review and should be submitted via Rapid Review. The cover letter should state whether the article was solicited and by whom.

Minireviews do not have abstracts. In the Abstract section of the submission form, put "Not applicable." The body of the Minireview may either have section headings or be set up like a short-form paper (see above).

Meeting Reviews

Meeting Reviews are brief summaries of recent scientific meetings that cover topics within the scope of AEM. Reviews should be timely and focus on major themes, new developments, emerging trends, and significant unanswered questions presented and discussed at the meeting. Sufficient background should be provided to make the report useful to the general reader. The author must provide written assurance from the relevant individuals that permission to cite their presented material has been granted.

Meeting Reviews, which may be solicited or proffered by authors, are subject to editorial review and should be submitted via Rapid Review.

Guest Commentaries

Guest Commentaries are communications written in response to invitations issued by the editors and concern relevant topics in microbiology that are not necessarily covered by Minireviews. They should raise issues of interest to the scholarly community, initiate or focus discussion, and propose needed position or consensus statements by the Academy of Microbiology, the National Academy of Sciences, and other leadership groups in research and education. Reviews of the literature, methods and other how-to papers, and responses targeted at a specific published paper are not appropriate. Guest Commentaries are subject to review.

The length may not exceed 4 printed pages, and the format is like that of a Minireview (see above). Commentaries should be submitted via Rapid Review.

Letters to the Editor

Letters to the Editor are intended only for comments on articles published previously in the journal and must cite published references to support the writer's argument.

Letters may be **no more than 500 words long and must be typed double spaced.** Refer to a recently published Letter for correct formatting. Note that authors and affiliations are listed at the foot of the Letter. Provide only the primary affiliation for each author.

All Letters to the Editor must be submitted electronically, and the manuscript type (Comment Letter) must be selected from the drop-down list in the submission form. The cover letter should state the volume and issue in which the article commented on was published, the title of the article, and the last name of the first author. In the Abstract section of the submission form, put "Not applicable." Letters to the Editor do not have abstracts. The Letter must have a title, which must appear on the manuscript and on the submission form. Figures and tables should be kept to a minimum.

The Letter will be sent to the editor who handled the article in question. If the editor believes that publication is warranted, he will solicit a reply from the corresponding author of the article and make a recommendation to the editor in chief. Final approval for publication rests with the editor in chief.

Please note that some indexing/abstracting services do not include Letters to the Editor in their databases.

Errata

The Erratum section provides a means of correcting errors that occurred during the writing, typing, editing, or printing (e.g., a misspelling, a dropped word or line, or mislabeling in a figure) of a published article. Send Errata directly to the ASM Journals Department (1752 N St., N.W., Washington, DC 20036-2904, USA), both on disk and in hard copy **(only one hard copy is necessary). Please see a recent issue for correct formatting.**

Authors' Corrections

The Author's Correction section provides a means of correcting errors of omission (e.g., author names or citations) and errors of a scientific nature that do not alter the overall basic results or conclusions of a published article.

For omission of an author's name, the authors of the article and the author whose name was inadvertently omitted must agree, in writing, to publication of the Correction. For other issues involving authorship, including contributions and use or ownership of data and/or materials, all disputing parties must agree, in writing, to publication of the Correction. Copies of the agreement letters must accompany the Correction and be sent directly to the Journals Department. Send the Correction both on disk and in hard copy **(only one hard copy is necessary). Please see a recent issue for correct formatting.**

Corrections of a scientific nature (e.g., an incorrect unit of measurement or order of magnitude used throughout; contamination of one of numerous cultures; or misidentification of a mutant strain, causing erroneous data for only a portion [noncritical] of the study) must be sent, both on disk and in hard copy, directly to the editor who handled the article and must be accompanied by *signed letters of agreement* from all of the authors of the article. If the editor believes that publication is warranted, he will send the Correction to the Journals Department for publication. *Note that the addition of new data is not permitted*

Retractions

Retractions are reserved for major errors or breaches of ethics that, for example, may call into question the source of the data or the validity of the results and conclusions of an article. Send a Retraction and an accompanying explanatory letter *signed by all of the authors* directly to the editor in chief of the journal. The editor who handled the paper and the chairman of the ASM Publications Board will be consulted. If all parties agree to the publication and content of the Retraction, it will be sent to the Journals Department for publication.

ILLUSTRATIONS AND TABLES

Digital files that are acceptable for production (see below) must be provided for all illustrations on return of the modified manuscript. (On initial submission, the entire paper may be submitted in PDF format.)

We strongly recommend that before returning their modified manuscripts, authors check the acceptability of their digital images for production by running their files through Rapid Inspector, a tool provided at the following URL: http://rapidinspector.cadmus.com/mw/. Rapid Inspector is an easy-to-use Web-based application that identifies file characteristics that may render the image unusable for production.

Illustrations may be continuous-tone images, line drawings, or composites. Color graphics may be submitted, but the cost of printing in color must be borne by the author. Suggestions about how to reduce costs and ensure accurate color reproduction are given below.

The preferred format for tables is MS Word; however, WordPerfect and Acrobat PDF are also acceptable (see the section on Tables below).

Since the contents of computer-generated images can be manipulated for better clarity, the Publications Board at its May 1992 meeting mandated that a description of the software/hardware used should be put in the figure legend(s).

Illustrations

File types and formats. As mentioned above, **illustrations may be supplied as PDF files for reviewing purposes only on initial submission; in fact, we recommend this option to minimize file upload time. At the modification stage, production quality digital files must be submitted:** TIFF or EPS files from supported applications or PowerPoint files (black and white only). Except for figures produced in PowerPoint, all graphics submitted with modified manuscripts must be bitmap, grayscale, or CMYK (*not* RGB). Acceptable file types and formats for production are given in the charts below. More-detailed instructions for preparing illustrations are available on the World Wide Web at http://cjs.cadmus.com/da. Please review this information before preparing your files. If you require additional information, please send an e-mail inquiry to digitalart@cadmus.com.

Minimum resolution. It is extremely important that a high enough resolution is used. Any imported images must be at the correct resolution before they are placed. Note, however, that the higher the resolution, the larger the file and the longer the upload time. Publication quality will *not* be improved by using a resolution higher than the minimum. Minimum resolutions are as follows:

300 dpi for grayscale and color
600 dpi for lettering
1,200 dpi for line art
600 dpi for combination art (lettering and images)

Size. All graphics **MUST be submitted at their intended publication size;** that is, the image uploaded should be 100% of its print dimensions so that no reduction or enlargement is necessary. Resolution must be at the required level at the submitted size. Include only the significant portion of an illustration. White space

Macintosh

Application	File type	
	Black and white	Color (CMYK)[a]
Adobe Illustrator 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 CS	EPS	EPS
Adobe InDesign 1.0	EPS	EPS
Adobe PageMaker 6.5	EPS	EPS
Adobe Photoshop 4.0, 5.0, 5.5, 6.0, 7.0, 8.0 CS	TIFF	TIFF
Adobe Photoshop 5.0 LE	TIFF	N/A[b]
ChemDraw Pro 5.0	EPS/TIFF	EPS/TIFF
Corel Photo-Paint 8.0	TIFF	EPS
CorelDRAW 6.0, 8.0	EPS/TIFF	EPS
Deneba Canvas 6.0, 7.0, 8.0	EPS/TIFF	EPS
Macromedia FreeHand 7.0, 8.0, 9.0	EPS	EPS
PowerPoint 98, 2001	PPT[c]	N/A[b]
Prism 3 by GraphPad	TIFF	N/A[b]
Synergy Kaleidagraph 3.08, 3.51	EPS	N/A[b]

[a] Color graphics must be saved and printed in the CMYK mode, *not* RGB.
[b] ASM accepts only black-and-white, not color, graphics created with Kaleidagraph, Adobe Photoshop 5.0 LE, Prism 3 by GraphPad, and PowerPoint.
[c] For instructions on saving PowerPoint files, refer to the Cadmus digital art website at http://cjs.cadmus.com/da/index.asp.

Windows

Application	File type	
	Black and white	Color (CMYK)[a]
Adobe Illustrator 7.0, 8.0, 9.0, 10.0, 11.0 CS	EPS	EPS
Adobe InDesign 1.0	EPS	EPS
Adobe PageMaker 6.5	EPS	EPS
Adobe Photoshop 4.0, 5.0, 5.5, 6.0, 7.0, 8.0 CS	TIFF	TIFF
Adobe Photoshop 5.0 LE	TIFF	N/A[b]
ChemDraw Pro 5.0	EPS/TIFF	EPS/TIFF
Corel Photo-Paint 8.0, 9.0	TIFF	EPS
CorelDRAW 7.0, 8.0, 9.0	EPS/TIFF	EPS
Deneba Canvas 6.0, 7.0	EPS/TIFF	EPS
Macromedia FreeHand 7.0, 8.0, 9.0	EPS	EPS
PowerPoint 97, 2000, XP	PPT[c]	N/A[b]
Prism 3 by GraphPad	TIFF	N/A[b]
SigmaPlot 8.01	EPS	EPS

[a] Color graphics must be saved and printed in the CMYK mode, *not* RGB.
[b] ASM accepts only black-and-white, not color, graphics created with Adobe Photoshop 5.0 LE, Prism 3 by GraphPad, and PowerPoint.
[c] For instructions on saving PowerPoint files, refer to the Cadmus digital art website at http://cjs.cadmus.com/da/index.asp.

must be cropped from the image, and excess space between panel labels and the image must be eliminated.

Maximum width for a 1-column figure: 3 5/16 inches (ca. 8.4 cm)
Maximum width for a 2-column figure: 6 7/8 inches (ca. 17.4 cm)
Minimum width for a 2-column figure: 4 3/4 inches (10.8 cm)
Maximum height: 9 1/16 inches (23.0 cm)

Contrast. Illustrations must contain sufficient contrast to withstand the inevitable loss of contrast and detail inherent in the printing process. See also the section on color illustrations below.

Labeling and assembly. All final lettering, labeling, tooling, etc., MUST be incorporated into the figures. It cannot be added at a later date. If a figure number is included, it must appear well outside the boundaries of the image itself. (Numbering may need to be changed at the copyediting stage.) Each figure must be uploaded as a separate file, and any multipanel figures must be assembled into one file; i.e., rather than uploading a separate file for each panel in a figure, assemble all panels in one piece and supply them as one file.

Fonts. To avoid font problems, set all type in one of the following fonts: Helvetica, Times Roman, European PI, Mathematical PI, or Symbol. All fonts other than these five must be converted to paths (or outlines) in the application with which they were created. For font use in PowerPoint images, refer to the Cadmus digital art website, http://cjs.cadmus.com/da.

Compression. Images created with Macintosh applications may be compressed with Stuffit. Images created with Windows applications may be compressed with WINZIP or PKZIP.

Color illustrations. Because the process of placing ink on paper by using printing presses is different from that used to produce a photo print or a laser print and the color rendition on images viewed on a monitor depends to some extent on monitor resolution, some differences in color and contrast between the image you submit and the image printed in the journal or published online will be evident. (Figures showing red or green fluorescence and those with a significant range of colors may be difficult or impossible to reproduce exactly.) Color illustrations must be saved as either TIFF or EPS files, according to the application used (see charts above). The mode of the TIFF or EPS file must be CMYK, *not* RGB. Graphics in the RGB color space are intended for display on a monitor only and will not separate correctly for printing.

The cost of printing in color must be borne by the author. The current color costs may be accessed from the submission form in Rapid Review and, for accepted manuscripts, will be included in the acceptance letter sent out by ASM. Adherence to the following guidelines, in addition to the general ones above, will help to minimize costs and to ensure color reproduction that is as accurate as possible.

Include only the significant portions of illustrations so that the number of printed pages containing color figures is minimized. The individual panels of a single figure must be assembled in a single file, including any necessary labels. Optimal color reproduction will be obtained if the composites comprise panels containing similar colors of similar lightness or darkness. If necessary, make unlike panels into separate figures/files; this will increase the cost, but the color rendition will be more accurate since the two panels will be "scanned" separately.

Drawings

Submit graphs, charts, complicated chemical or mathematical formulas, diagrams, and other drawings as finished products not requiring additional artwork or typesetting. No part of the graph or drawing may be handwritten. *All* elements, including letters, numbers, and symbols, *must* be easily readable, and both axes of a graph must be labeled. Keep in mind that the journal is published both in print and online and that the same electronic files submitted by the authors are used to produce both.

When creating line art, please use the following guidelines:

1. **All art MUST be submitted at its intended publication size.** For acceptable dimensions, see the Size section on p. 12.
2. **Avoid using screens (i.e., shading)** in line art. It can be difficult and time-consuming to reproduce these images without moiré patterns. Various pattern backgrounds are preferable to screens as long as the patterns are not imported from another application. If you must use images containing screens,
   - Generate the image at line screens of 85 lines per inch or lower.
   - When applying multiple shades of gray, differentiate the gray levels by at least 20%.
   - Never use levels of gray below 20% or above 70% as they will fade out or become totally black upon scanning and reduction.
3. Use thick, solid lines that are no finer than 1 point in thickness.
4. No type should be smaller than 6 points at the final publication size.
5. Avoid layering type directly over shaded or textured areas.
6. Avoid the use of reversed type (white lettering on a black background).
7. Avoid heavy letters, which tend to close up, and unusual symbols, which the printer may not be able to reproduce in the legend.
8. If colors are used, avoid using similar shades of the same color and avoid very light colors.

In figure ordinate and abscissa scales (as well as table column headings), **avoid the ambiguous use of numbers with exponents.** Usually, it is preferable to use the Système International d'Unités (SI) symbols (μ for 10^{-6}, m for 10^{-3}, k for 10^{3}, M for 10^{6}, etc.). A complete listing of SI symbols can be found in the International Union of Pure and Applied Chemistry (IUPAC) "Manual of Symbols and Terminology for Physicochemical Quantities and Units" (Pure Appl. Chem. **21**:3–44, 1970). Thus, a representation of 20,000 cpm on a figure ordinate is to be made by the number 20 accompanied by the label kcpm.

When powers of 10 must be used, the journal requires that the exponent power be associated with the number shown. In representing 20,000 cells per ml, the numeral on the ordinate would be "2" and the label would be "10^{4} cells per ml" (not "cells per ml $\times$ 10^{-4}"). Likewise, an enzyme activity of 0.06 U/ml would be shown as 6 accompanied by the label 10^{-2} U/ml. The preferred designation would be 60 mU/ml (milliunits per milliliter).

Presentation of Nucleic Acid Sequences

Nucleic acid sequences of limited length which are the primary subject of a study may be presented freestyle in the most effective format. Longer nucleic acid sequences must be presented as figures in the following format to conserve space. Print the sequence in lines of approximately 100 to 120 nucleotides in a nonproportional (monospace) font that is easily legible when published with a line length of 6 inches (ca. 15.2 cm). If possible, lines of nucleic acid sequence should be further subdivided into blocks of 10 or 20 nucleotides by spaces within the sequence or by marks above it. Uppercase and lowercase letters may be used to designate the exon-intron structure, transcribed regions, etc., if the lowercase letters remain legible at a 6-inch (ca. 15.2-cm) line length. Number the sequence line by line; place numerals, representing the first base of each line, to the left of the lines. **Minimize spacing between lines of sequence, leaving room only for annotation of the sequence.** Annotation may include boldface, underlining, brackets, boxes, etc. Encoded amino acid sequences may be presented, if necessary, immediately above or below the first nucleotide of each codon, by using the single-letter amino acid symbols. Comparisons of multiple nucleic acid sequences should conform as nearly as possible to the same format.

Figure Legends

Legends should provide enough information so that the figure is understandable without frequent reference to the text. However, detailed experimental methods must be described in the Materials and Methods section, not in a figure legend. A method that is unique to one of several experiments may be reported in a legend only if the discussion is very brief (one or two sentences). Define all symbols used in the figure and define all abbreviations that are not used in the text.

Tables

Tables that contain artwork, chemical structures, or shading must be submitted as illustrations in an acceptable format at the modification stage. The preferred format for regular tables is MS Word; however, WordPerfect and Acrobat PDF are also acceptable. Note that a straight Excel file is *not* currently an acceptable format. Excel files must be either embedded in a Word or WordPerfect document or converted to PDF *before* being uploaded. **If your modified manuscript contains PDF tables, select "for reviewing purposes only" at the beginning of the file upload process.**

Tables should be formatted as follows. Arrange the data so that **columns of like material read down, not across.** The headings should be sufficiently clear so that the meaning of the data is understandable without reference to the text. See the Abbreviations section (p. 18) of these Instructions for those that should be used in tables. Explanatory footnotes are acceptable, but more extensive table "legends" are not. Footnotes should not include detailed descriptions of the experiment. Tables must include enough information to warrant table format; those with fewer than six pieces of data will be incorporated into the text by the copy editor. Table 1 is an example of a well-constructed table.

TABLE 1. Distribution of protein and ATPase in fractions of dialyzed membranes[a]

Membrane	Fraction	ATPase	
		U/mg of protein	Total U
Control	Depleted membrane	0.036	2.3
	Concentrated supernatant	0.134	4.82
E1 treated	Depleted membrane	0.034	1.98
	Concentrated supernatant	0.11	4.6

[a] Specific activities of ATPase of nondepleted membranes from control and treated bacteria were 0.21 and 0.20, respectively.

Cover Photographs and Drawings

AEM publishes photographs and drawings on the front cover. Invitations are issued to authors whose manuscripts are returned for modification or whose manuscripts have been accepted for publication in AEM; material should be related to the work presented in the AEM manuscript. Unsolicited photos will be considered in hard-copy format (two copies) only; if an unsolicited photo is chosen for the cover, the author may be asked to submit digital files. No material submitted for consideration will be returned to the author. Authors will be notified only if their cover art is selected. Copyright for the chosen material must be transferred to ASM. A short description of the cover material will be included at the end of the table of contents or the author index of the issue. Technical specifications for submission are available from the cover editor, Matthew R. Parsek (e-mail: matthew-parsek@uiowa.edu).

NOMENCLATURE

Chemical and Biochemical Nomenclature

The recognized authority for the names of chemical compounds is *Chemical Abstracts* (CAS, Columbus, Ohio) and its indexes. *The Merck Index*, 13th ed. (Merck & Co., Inc., Whitehouse Station, N.J., 2001), is also an excellent source. For biochemical terminology, including abbreviations and symbols, consult *Biochemical Nomenclature and Related Documents* (1978; reprinted for The Biochemical Society, London, England) and the instructions to authors of the *Journal of Biological Chemistry* and the *Archives of Biochemistry and Biophysics* (first issues of each year).

Do not express molecular weight in daltons; molecular weight is a unitless ratio. Molecular mass is expressed in daltons.

For enzymes, use the recommended (trivial) name assigned by the Nomenclature Committee of the International Union of Biochemistry (IUB) as described in *Enzyme Nomenclature* (Academic Press, Inc., New York, N.Y., 1992) and at http://www.chem.qmul.ac.uk/iubmb/enzyme/. If a nonrecommended name is used, place the proper (trivial) name in parentheses at first use in the abstract and text. Use the EC number when one has been assigned, and express enzyme activity either in katals (preferred) or in the older system of micromoles per minute.

Nomenclature of Microorganisms

Binary names, consisting of a generic name and a specific epithet (e.g., *Escherichia coli*), must be used for all microorganisms. Names of categories at or above the genus level may be used alone, but specific and subspecific epithets may not. A specific epithet must be preceded by a generic name, written out in full the first time it is used in a paper. Thereafter, the generic name should be abbreviated to the initial capital letter (e.g., *E. coli*), provided there can be no confusion with other genera used in the paper. Names of all taxa (kingdoms, phyla, classes, orders, families, genera, species, and subspecies) are printed in italics and should be underlined (or italicized) in the manuscript; strain designations and numbers are not. Vernacular (common) names should be in lowercase roman type (e.g., streptococcus, brucella). For *Salmonella*, genus, species, and subspecies names should be rendered in standard form: *Salmonella enterica* at first use, *S. enterica* thereafter; *Salmonella enterica* subsp. *arizonae* at first use, *S. enterica* subsp. *arizonae* thereafter. Names of serovars should be in roman type with the first letter capitalized: *Salmonella enterica* serovar Typhimurium. After the first use, the serovar may also be given without a species name: *Salmonella* serovar Typhimurium. For other information regarding serovar designations, see *Identification and Serotyping of Salmonella and an Update of the Kaufmann-White Scheme* (A. C. McWhorter-Murlin and F. W. Hickman-Brenner, Centers for Disease Control and Prevention, Atlanta, Ga., 1994) and *Antigenic Formulas of the Salmonella Serovars* (M. Y. Popoff and L. Le Minor, WHO Collaborating Centre for Reference and Research on *Salmonella*, Institut Pasteur, Paris, France, 1997). For a summary of the current standards for *Salmonella* nomenclature and the Kaufmann-White criteria, see the articles by Brenner et al. (J. Clin. Microbiol. 38:2465–2467, 2000) and McQuiston et al. (J. Clin. Microbiol. 42:1923–1932, 2004).

The spelling of bacterial names should follow the *Approved Lists of Bacterial Names (Amended) & Index of the Bacterial and Yeast Nomenclatural Changes* (V. B. D. Skerman et al. ed., ASM Press, Washington, D.C., 1989) and the validation lists and notification lists published in the *International Journal of Systematic and Evolutionary Microbiology* (formerly the *Inter-*

national Journal of Systematic Bacteriology) since January 1989. In addition, two sites on the World Wide Web list current approved bacterial names: Bacterial Nomenclature Up-to-Date (http://www.dsmz.de/microorganisms/main.php?contentleft_id=14) and List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.cict.fr). If there is reason to use a name that does not have standing in nomenclature, the name should be enclosed in quotation marks in the title and at its first use in the abstract and the text and an appropriate statement concerning the nomenclatural status of the name should be made in the text. "*Candidatus*" species should always be set in quotation marks.

For guidelines regarding new names and descriptions of new genera and species, see the articles by Tindall (Int. J. Syst. Bacteriol. 49:1309–1312, 1999) and Stackebrandt et al. (Int. J. Syst. Evol. Microbiol. 52:1043–1047, 2002). To validate new names and/or combinations, authors must submit three copies of their published article to the *International Journal of Systematic and Evolutionary Microbiology*.

It is recommended that a strain be deposited in at least two recognized culture collections in different countries when that strain is necessary for the description of a new taxon (Int. J. Syst. Evol. Microbiol. 50:2239–2244, 2000).

Since the classification of fungi is not complete, it is the responsibility of the author to determine the accepted binomial for a given organism. Sources for these names include *The Yeasts: a Taxonomic Study*, 4th ed. (C. P. Kurtzman and J. W. Fell, ed., Elsevier Science Publishers B.V., Amsterdam, The Netherlands, 1998), and *Ainsworth and Bisby's Dictionary of the Fungi*, 9th ed. (P. M. Kirk, P. F. Cannon, J. C. David, and J. A. Stalpers, ed., CABI Publishing, Wallingford, Oxfordshire, United Kingdom, 2001).

Names used for viruses should be those approved by the International Committee on Taxonomy of Viruses (ICTV) and published in *Virus Taxonomy: Classification and Nomenclature of Viruses, Seventh Report of the International Committee on Taxonomy of Viruses* (M. H. V. van Regenmortel et al., ed., Academic Press, San Diego, Calif., 2000). In addition, the recommendations of the ICTV regarding the use of species names should generally be followed: when the entire species is discussed as a taxonomic entity, the species name, like other taxa, is italic and has the first letter and any proper nouns capitalized (e.g., *Tobacco mosaic virus, Murray Valley encephalitis virus*). When the behavior or manipulation of individual viruses is discussed, the vernacular (e.g., tobacco mosaic virus, Murray Valley encephalitis virus) should be used. If desired, synonyms may be added parenthetically when the name is first mentioned. Approved generic (or group) and family names may also be used.

Microorganisms, viruses, and plasmids should be given designations consisting of letters and serial numbers. It is generally advisable to include a worker's initials or a descriptive symbol of locale, laboratory, etc., in the designation. Each new strain, mutant, isolate, or derivative should be given a new (serial) designation. This designation should be distinct from those of the genotype and phenotype, and genotypic and phenotypic symbols should not be included. Plasmids are named with a lowercase "p" followed by the designation in uppercase letters and numbers. To avoid the use of the same designation as that of a widely used strain or plasmid, check the designation against a publication database such as Medline.

Genetic Nomenclature

To facilitate accurate communication, **it is important that standard genetic nomenclature be used whenever possible and that deviations or proposals for new naming systems be endorsed by an appropriate authoritative body.** Review and/or publication of submitted manuscripts that contain new or nonstandard nomenclature may be delayed by the editor or the Journals Department so that they may be reviewed by the Genetics and Genomics Committee of the ASM Publications Board.

Before submission of manuscripts, authors may direct questions on genetic nomenclature to the committee's chairman: Maria Costanzo (e-mail: maria@genome.stanford.edu). Such a consultation should be mentioned in the manuscript submission letter.

Bacteria. The genetic properties of bacteria are described in terms of phenotypes and genotypes. The phenotype describes the observable properties of an organism. The genotype refers to the genetic constitution of an organism, usually in reference to some standard wild type. The guidelines that follow are based on the recommendations of Demerec et al. (Genetics 54:61–76, 1966).

(i) Phenotypic designations must be used when mutant loci have not been identified or mapped. They can also be used to identify the protein product of a gene, e.g., the OmpA protein. Phenotypic designations generally consist of three-letter symbols; these are *not* italicized, and the first letter of the symbol is capitalized. It is preferable to use Roman or Arabic numerals (instead of letters) to identify a series of related phenotypes. Thus, a series of nucleic acid polymerase mutants might be designated Pol1, Pol2, Pol3, etc. Wild-type characteristics can be designated with a superscript plus (Pol^+), and, when necessary for clarity, negative superscripts (Pol^-) can be used to designate mutant characteristics. Lowercase superscript letters may be used to further delineate phenotypes (e.g., Str^r for streptomycin resistance). Phenotypic designations should be defined.

(ii) Genotypic designations are also indicated by three-letter locus symbols. In contrast to phenotypic designations, these are lowercase italic (e.g., *ara his rps*). If several loci govern related functions, these are distinguished by italicized capital letters following the locus symbol (e.g., *araA araB araC*). Promoter, terminator, and operator sites should be indicated as described by Bachmann and Low (Microbiol. Rev. 44:1–56, 1980), e.g., *lacZp*, *lacAt*, and *lacZo*.

(iii) Wild-type alleles are indicated with a superscript plus (ara^+ his^+). A superscript minus is not used to indicate a mutant locus; thus, one refers to an *ara* mu-

tant rather than an *ara*$^-$ strain.

(iv) Mutation sites are designated by placing serial isolation numbers (allele numbers) after the locus symbol (e.g., *araA1 araA2*). If it is not known in which of several related loci the mutation has occurred, a hyphen is used instead of the capital letter (e.g., *ara-23*). It is essential in papers reporting the isolation of new mutants that allele numbers be given to the mutations. For *Escherichia coli*, there is a registry of such numbers: *E. coli* Genetic Stock Center, Department of Biology, Yale University, New Haven, CT 06511-5188. For the genus *Salmonella*, the registry is *Salmonella* Genetic Stock Center, Department of Biology, University of Calgary, Calgary, Alberta T2N 1N4, Canada. For the genus *Bacillus*, the registry is *Bacillus* Genetic Stock Center, Ohio State University, Columbus, OH 43210.

(v) The use of superscripts with genotypes (other than + to indicate wild-type alleles) should be avoided. Designations indicating amber mutations (Am), temperature-sensitive mutations (Ts), constitutive mutations (Con), cold-sensitive mutations (Cs), production of a hybrid protein (Hyb), and other important phenotypic properties should follow the allele number [e.g., *araA230*(Am) *hisD21*(Ts)]. All other such designations of phenotype must be defined at the first occurrence. If superscripts *must* be used, they must be approved by the editor and defined at the first occurrence in the text.

Subscripts may be used in two situations. Subscripts may be used to distinguish between genes (having the same name) from different organisms or strains, e.g., $his_{E.\ coli}$ or $his_{K\text{-}12}$ for the *his* genes of *E. coli* or strain K-12 in another species or strain, respectively. An abbreviation may also be used if it is explained. Similarly, a subscript is also used to distinguish between genetic elements that have the same name. For example, the promoters of the *gln* operon can be designated $glnAp_1$ and $glnAp_2$. This form departs slightly from that recommended by Bachmann and Low (e.g., *desC1p*).

(vi) Deletions are indicated by the symbol Δ placed before the deleted gene or region, e.g., Δ*trpA432*, Δ(*aroP-aceE*)*419*, or Δ*his*(*dhuA hisJ hisQ*)*1256*. Similarly, other symbols can be used (with appropriate definition). Thus, a fusion of the *ara* and *lac* operons can be shown as Φ(*ara-lac*)*95*. Likewise, Φ(*araB'-lacZ*$^+$)*96* indicates that the fusion results in a truncated *araB* gene fused to an intact *lacZ* gene, and Φ(*malE-lacZ*)*97*(Hyb) shows that a hybrid protein is synthesized. An inversion is shown as IN(*rrnD- rrnE*)*1*. An insertion of an *E. coli his* gene into plasmid pSC101 at zero kilobases (0 kb) is shown as pSC101 Ω(0kb::K-12*hisB*)*4*. An alternative designation of an insertion can be used in simple cases, e.g., *galT236* ::Tn*5*. The number *236* refers to the locus of the insertion, and if the strain carries an additional *gal* mutation, it is listed separately. Additional examples, which utilize a slightly different format, can be found in the papers by Campbell et al. and Novick et al. cited below. It is important in reporting the construction of strains in which a mobile element was inserted and subsequently deleted that this fact be noted in the strain table. This can be done by listing the genotype of the strain used as an intermediate in a table footnote or by making a direct or parenthetical remark in the genotype, e.g., (F$^-$), ΔMu cts, or *mal*::ΔMu cts::*lac*. In setting parenthetical remarks within the genotype or dividing the genotype into constituent elements, parentheses and brackets are used without special meaning; brackets are used outside parentheses. To indicate the presence of an episome, parentheses (or brackets) are used (λ, F$^+$). Reference to an integrated episome is indicated as described above for inserted elements, and an exogenote is shown as, for example, W3110/F′8(*gal*$^+$).

For information about the symbols in current use, consult Berlyn (Microbiol. Mol. Biol. Rev. **62**:814–984, 1998) for *E. coli* K-12, Sanderson and Roth (Microbiol. Rev. **52**:485–532, 1988) for *Salmonella* serovar Typhimurium, Holloway et al. (Microbiol. Rev. **43**:73–102, 1979) for the genus *Pseudomonas*, Piggot and Hoch (Microbiol. Rev. **49**:158–179, 1985) for *Bacillus subtilis*, Perkins et al. (Microbiol. Rev. **46**:426–570, 1982) for *Neurospora crassa*, and Mortimer and Schild (Microbiol. Rev. **49**:181–213, 1985) for *Saccharomyces cerevisiae*. For yeasts, *Chlamydomonas* spp., and several fungal species, symbols such as those given in the *Handbook of Microbiology* (A. I. Laskin and H. A. Lechevalier, ed., CRC Press, Inc., Cleveland, Ohio, 1974) should be used.

Conventions for naming genes. It is recommended that (entirely) new genes be given names that are mnemonics of their function, avoiding names that are already assigned and earlier or alternative gene names, irrespective of the bacterium for which such assignments have been made. Similarly, it is recommended that, whenever possible, homologous genes present in different organisms receive the same name. When homology is not apparent or the function of a new gene has not been established, a provisional name may be given by one of the following methods. (i) The gene may be named on the basis of its map location in the style *yaaA*, analogous to the style used for recording transposon insertions (*zef*) as discussed below. A list of such names in use for *E. coli* has been published by Rudd (Microbiol. Mol. Biol. Rev. **62**: 985–1019, 1998). (ii) A provisional name may be given in the style described by Demerec et al. (e.g., *usg*, gene upstream of *folC*). Such names should be unique, and names such as *orf* or *genX* should not be used. For reference, the *E. coli* Genetic Stock Center's database includes an updated listing of *E. coli* gene names and gene products. It is accessible on the Internet (http://cgsc.biology.yale.edu/cgsc.html). The Center's relational database can also be searched via Telnet; for access, send a request to berlyn@cgsc.biology.yale.edu. A list can also be found in the work of Riley (Microbiol. Rev. **57**:862–952, 1993). For the genes of other bacteria, consult the references given above.

"Mutant" versus "mutation." Keep in mind the distinction between a *mutation* (an alteration of the primary sequence of the genetic material) and a *mutant* (a strain carrying one or more mutations). One may speak about the mapping of a mutation, but one cannot map a mutant. Likewise, a mutant has no genetic locus, only a phenotype.

"Homology" versus "similarity." For use of terms that describe relationships between genes, consult the articles by Theissen (Nature **415**:741, 2002) and Fitch (Trends Genet. **16**:227–231, 2000). "Homology" implies a relationship between genes that share a common evolutionary origin; partial homology is not recognized. When sequence comparisons are discussed, it is more appropriate to use the term "percent sequence similarity" or "percent sequence identity," as appropriate.

Strain designations. Do not use a genotype as a name (e.g., "subsequent use of *leuC6* for transduction"). If a strain designation has not been chosen, select an appropriate word combination (e.g., "another strain containing the *leuC6* mutation").

"Natural" versus "artificial" transformation. Natural transformation is a process whereby the recipient cell has the inherent capacity to take up and integrate exogenous DNA into its genome. As such, natural transformation is part of the biology of the recipient cell line and should not be confused with processes through which integration of DNA is forced upon recipient cells.

Viruses. The genetic nomenclature for viruses differs from that for bacteria. In most instances, viruses have no phenotype, since they have no metabolism outside host cells. Therefore, distinctions between phenotype and genotype cannot be made. Superscripts are used to indicate hybrid genomes. Genetic symbols may be one, two, or three letters. For example, a mutant strain of λ might be designated λ *A*am11 *int*2 *red*114 *c*I857; this strain carries mutations in genes cI, *int*, and *red* and an amber-suppressible (am) mutation in gene *A*. A strain designated λ att^{434} imm^{21} would represent a hybrid of phage λ which carries the immunity region (*imm*) of phage 21 and the attachment (*att*) region of phage 434. Host DNA insertions into viruses should be delineated by square brackets, and the genetic symbols and designations for such inserted DNA should conform to those used for the host genome. Genetic symbols for phage λ can be found in reports by Szybalski and Szybalski (Gene **7**:217–270, 1979) and Echols and Murialdo (Microbiol. Rev. **42**:577–591, 1978).

Eukaryotes. For information about the genetic nomenclature of eukaryotes, see the Instructions to Authors for *Eukaryotic Cell* and *Molecular and Cellular Biology*.

Transposable elements, plasmids, and restriction enzymes. Nomenclature of transposable elements (insertion sequences, transposons, phage Mu, etc.) should follow the recommendations of Campbell et al. (Gene **5**: 197–206, 1979), with the modifications given in section vi above. The Internet site where insertion sequences of eubacteria and archaea are described and new sequences can be recorded is http://www-is.biotoul.fr/is.html.

The system of designating transposon insertions at sites where there are no known loci, e.g., *zef-123*::Tn*5*, has been described by Chumley et al. (Genetics **91**:639–655, 1979). The nomenclature recommendations of Novick et al. (Bacteriol. Rev. **40**:168–189, 1976) for plasmids and plasmid-specified activities, of Low (Bacteriol. Rev. **36**:587–607, 1972) for F′ factors, and of Roberts et al. (Nucleic Acids Res. **31**:1805–1812, 2003) for restriction enzymes, DNA methyltransferases, homing endonucleases, and their genes should be used when possible. The nomenclature for recombinant DNA molecules constructed in vitro follows the nomenclature for insertions in general. DNA inserted into recombinant DNA molecules should be described by using the gene symbols and conventions for the organism from which the DNA was obtained.

Tetracycline resistance determinants. The nomenclature for tetracycline resistance determinants is based on the proposal of Levy et al. (Antimicrob. Agents Chemother. **43**:1523–1524, 1999). The style for such determinants is, e.g., Tet B; the space helps distinguish the determinant designation from that for phenotypes and proteins (TetB). The above-referenced article shows the correct format for genes, proteins, and determinants in this family.

ABBREVIATIONS AND CONVENTIONS

Verb Tense

ASM strongly recommends that for clarity you use the past tense to narrate particular events in the past, including the procedures, observations, and data of the study that you are reporting. Use the present tense for your own general conclusions, the conclusions of previous researchers, and generally accepted facts. Thus, most of the abstract, Materials and Methods, and Results will be in the past tense, and most of the introduction and some of the Discussion will be in the present tense.

Be aware that it may be necessary to vary the tense in a single sentence. For example, it is correct to say "White (30) demonstrat*ed* that XYZ cells *grow* at pH 6.8," "Figure 2 shows that ABC cells fail*ed* to grow at room temperature," and "Air *was* removed from the chamber and the mice *died*, which *proves* that mice *require* air." In reporting statistics and calculations, it is correct to say "The values for the ABC cells *are* statistically significant, indicating that the drug inhibit*ed*. . . ."

For an in-depth discussion of tense in scientific writing, see p. 207–209 in *How To Write and Publish a Scientific Paper*, 5th ed.

Abbreviations

General. Abbreviations should be used as an aid to the reader rather than as a convenience to the author, and therefore their **use should be limited.** Abbreviations other than those recommended by the IUPAC-IUB (*Biochemical Nomenclature and Related Documents*, 1978) should be used only when a case can be made for necessity, such as in tables and figures.

It is often possible to use pronouns or to paraphrase a long word after its first use (e.g., "the drug" or "the substrate"). Standard chemical symbols and trivial names or

their symbols (folate, Ala, Leu, etc.) may also be used.

It is strongly recommended that all abbreviations except those listed below be introduced in the first paragraph in Materials and Methods. Alternatively, define each abbreviation and introduce it in parentheses the first time it is used; e.g., "cultures were grown in Eagle minimal essential medium (MEM)." Generally, eliminate abbreviations that are not used at least three times in the text (including tables and figure legends).

Not requiring introduction. In addition to abbreviations for Système International d'Unités (SI) units of measurement, other common units (e.g., bp, kb, and Da), and chemical symbols for the elements, the following should be used without definition in the title, abstract, text, figure legends, and tables: DNA (deoxyribonucleic acid); cDNA (complementary DNA); RNA (ribonucleic acid); cRNA (complementary RNA); RNase (ribonuclease); DNase (deoxyribonuclease); rRNA (ribosomal RNA); mRNA (messenger RNA); tRNA (transfer RNA); AMP, ADP, ATP, dAMP, ddATP, GTP, etc. (for the respective 5′ phosphates of adenosine and other nucleosides) (add 2′-, 3′-, or 5′- when needed for contrast); ATPase, dGTPase, etc. (adenosine triphosphatase, deoxyguanosine triphosphatase, etc.); NAD (nicotinamide adenine dinucleotide); NAD^+ (nicotinamide adenine dinucleotide, oxidized); NADH (nicotinamide adenine dinucleotide, reduced); NADP (nicotinamide adenine dinucleotide phosphate); NADPH (nicotinamide adenine dinucleotide phosphate, reduced); $NADP^+$ (nicotinamide adenine dinucleotide phosphate, oxidized); poly(A), poly(dT), etc. (polyadenylic acid, polydeoxythymidylic acid, etc.); oligo(dT), etc. (oligodeoxythymidylic acid, etc.); UV (ultraviolet); PFU (plaque-forming units); CFU (colony-forming units); MIC (minimal inhibitory concentration); Tris [tris(hydroxymethyl)aminomethane]; DEAE (diethylaminoethyl); EDTA (ethylenediaminetetraacetic acid); EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]; HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid); PCR (polymerase chain reaction); and AIDS (acquired immunodeficiency syndrome). Abbreviations for cell lines (e.g., HeLa) also need not be defined.

The following abbreviations should be used without definition in tables:

amt (amount)
approx (approximately)
avg (average)
concn (concentration)
diam (diameter)
expt (experiment)
exptl (experimental)
ht (height)
mo (month)
mol wt (molecular weight)
no. (number)
prepn (preparation)
SD (standard deviation)
SE (standard error)
SEM (standard error of the mean)
sp act (specific activity)
sp gr (specific gravity)
temp (temperature)
tr (trace)
vol (volume)
vs (versus)
wk (week)
wt (weight)
yr (year)

Reporting Numerical Data

Standard metric units are used for reporting length, weight, and volume. For these units and for molarity, use the prefixes m, μ, n, and p for 10^{-3}, 10^{-6}, 10^{-9}, and 10^{-12}, respectively. Likewise, use the prefix k for 10^3. Avoid compound prefixes such as mμ or μμ. Parts per million (ppm) may be used when that is the common measure for the science in that field. Units of temperature are presented as follows: 37°C or 324 K.

When fractions are used to express such units as enzymatic activities, it is preferable to use whole units, such as g or min, in the denominator instead of fractional or multiple units, such as μg or 10 min. For example, "pmol/min" is preferable to "nmol/10 min," and "μmol/g" is preferable to "nmol/μg." It is also preferable that an unambiguous form, such as exponential notation, be used; for example, "μmol g^{-1} min^{-1}" is preferable to "μmol/g/min." Always report numerical data in the applicable SI units.

Representation of data as accurate to more than two significant figures must be justified by presentation of appropriate statistical analyses.

For a review of some common errors associated with statistical analyses and reports, plus guidelines on how to avoid them, see the article by Olsen (Infect. Immun. 71:6689–6692, 2003).

For a review of basic statistical considerations for virology experiments, see the article by Richardson and Overbaugh (J. Virol. 79:669–676, 2005).

Statistics

If biological variation within a treatment (coefficient of variation, the standard deviation divided by the mean) is small (less than 10%) and the difference among treatment means is large (greater than 3 standard deviations), it is not necessary to report statistics. If the data do not meet these criteria, however, the authors must include an appropriate statistical analysis (e.g., Student's *t* test, analysis of variance, Tukey's test, etc.). Statistics should represent the variation among biological units (e.g., replicate incubations) and not just the variation due to method of analysis.

Phylogenetic trees based on nucleotide or amino acid sequence alignments must be supported by appropriate statistical analyses of tree stability (e.g., bootstrap analysis), and nonsupported branches (e.g., bootstrap coefficients below 50%) should be collapsed. A copy of the alignment should be available for examination by the editor or the reviewers upon request.

For a review of some common errors associated with statistical analyses and reports, plus guidelines on how to avoid them, see the article by Olsen (Infect. Immun. 71:6689–6692, 2003).

For a review of basic statistical considerations for virology experiments, see the article by Richardson and Overbaugh (J. Virol. 79:669–676, 2005).

Equations

In mathematical equations, indicate the order of operations clearly by enclosing operations in parentheses, brackets, and braces, in that order: $(a + b) \times c$ or $a + (b \times c)$, $100 \times \{[(a/b) \times c] + d\}$ or $100 \times \{a/[(b \times c) + d]\}$. Italicize (by underlining) variables and constants (but not numerals), and use roman type for designations: E_o, E_n, M_r, K_m, K_s, $a + 2b = 1.2$ mM, Ca^{2+} $V_{max} = \exp(1.5x + y)$, BOD $= 2.7x^2$.

Isotopically Labeled Compounds

For simple molecules, isotopic labeling is indicated in the chemical formula (e.g., $^{14}CO_2$, $^3H_2$, and $H^{35}SO_4$). Brackets are not used when the isotopic symbol is attached to the name of a compound that in its natural state does not contain the element (e.g., ^{32}S-ATP) or to a word that is not a specific chemical name (e.g., ^{131}I-labeled protein, ^{14}C-amino acids, and ^{3}H-ligands).

For specific chemicals, the symbol for the isotope introduced is placed in brackets directly preceding the part of the name that describes the labeled entity. Note that configuration symbols and modifiers precede the isotopic symbol. The following examples illustrate correct usage.

[^{14}C]urea	UDP-[U-^{14}C]glucose
L-[*methyl*-^{14}C]methionine	*E. coli* [^{32}P]DNA
[2,3-^{3}H]serine	fructose 1,6-[1-^{32}P]bisphosphate
[α-^{14}C]lysine	
[γ-^{32}P]ATP	

AEM follows the same conventions for isotopic labeling as the *Journal of Biological Chemistry*, and more-detailed information can be found in the instructions to authors of that journal (first issue of each year).