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ANÁLISIS PROTEOMICO DEL BIOFILM MIXTO *Candida albicans-Pseudomonas aeruginosa*.

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DEDICATORIA

Dedico esta tesis con mucho amor a mis padres, mi hijo y esposo.

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ÍNDICE

1. RESUMEN

La investigación de las interacciones microbio-microbio es esencial para entender las actividades *in vivo* de microorganismos comensales y patogénicos. *Candida albicans*, un hongo polimórfico, y *Pseudomonas aeruginosa*, una bacteria Gram-negativa, son dos patógenos oportunistas que interactúan en varias infecciones polimicrobianas en humanos. Para determinar cómo *P. aeruginosa* afecta la fisiología de *C. albicans* y viceversa, comparamos el proteoma de cada especie en biofilms mixtos *versus* biofilms de una sola especie. Además, realizamos un análisis de proteínas extracelulares. Observamos que en biofilms mixtos, ambas especies expresan diferencialmente proteínas de virulencia, proteínas asociadas a resistencia a drogas, proteasas y defensa celular, proteínas de estrés y proteínas reguladas por hierro. Además en biofilm mixto, ambas especies despliegan un incremento en mutabilidad comparado a los biofilms monoespecíficos. Esta característica fue correlacionada con la represión de enzimas que confieren protección contra la oxidación del ADN. En los biofilms mixtos la bacteria incrementó la producción de factores de virulencia (pioverdina, rhamnolipidos y piocianina), los cuales son utilizados por la bacteria para causar enfermedad. En conjunto, nuestros resultados indican que la competencia entre patógenos oportunistas incrementa la producción de factores de virulencia y así, puede alterar el curso de las interacciones hospedero-patógeno en las infecciones polimicrobianas.

2. ABSTRACT

The investigation of microbe-microbe interactions is essential for understanding the *in vivo* activities of commensal and pathogenic microorganisms. *Candida albicans*, a polymorphic fungus, and *Pseudomonas aeruginosa*, a Gram-negative bacterium, are two opportunistic pathogens that interact in various polymicrobial infections in humans. To determine how *P. aeruginosa* affects the physiology of *C. albicans* and *vice versa*, we compared the proteomes of each species in mixed biofilms *versus* single-species biofilms. In addition, extracellular proteins were analyzed. We observed that in mixed biofilms, both species showed differential expression of virulence proteins, multidrug resistance-associated proteins, proteases and cell defense, stress and iron-regulated proteins. Furthermore, in mixed biofilms, both species displayed an increase in mutability compared to monospecific biofilms. This characteristic was correlated with the downregulation of enzymes conferring protection against DNA oxidation. In mixed biofilms, the bacterium increased the production of virulence factors (pyoverdine, rhamnolipids and pyocyanin), which are used by the bacteria to cause disease. Overall, our results indicate that interspecies competition between these opportunistic pathogens enhances the production of virulence factors and increases mutability and, thus, can alter the course of host-pathogen interactions in polymicrobial infections.

3. INTRODUCCIÓN

Las comunidades microbianas son un componente integral y funcional de la mayoría de ambientes y sistemas biológicos (Eckburg *et al*., 2005). Una vista emergente del mundo microbiano es aquella en la cual bacterias ejercen su influencia dentro de comunidades a través de la producción, censado y respuesta a un arreglo de señales químicas. Inherente a la función de cualquier comunidad es la necesidad para que la información sea transferida entre individuos para coordinar comportamientos, basados en interacciones cooperativas o competitivas (Diggle *et al.,* 2007). Los microbios sobreviven en el ambiente por el intercambio de nutrientes, productos de desecho y de información química.

A pesar de las diferencias con respecto a la fisiología de organismos multicelulares, las bacterias y otros microbios organizan comunidades capaces de ejecutar funciones parecidas a las de un órgano en un determinado nicho. Los biofilms ejemplifican este concepto, en el que un ensamble de células y la producción de una matriz extracelular le permite a las células en una población realizar distintos comportamientos de manera coordinada a través de una población completa.

Las interacciones entre bacterias y hongos pueden tener efectos importantes en la sobrevivencia, colonización y patogénesis de los organismos (Figura 1). En algunas situaciones las bacterias proveen compuestos que incrementan la producción de determinantes de virulencia de algunos hongos patógenos (Wargo & Hogan, 2006). Por el contrario otras bacterias pueden inhibir la virulencia de hongos afectando su capacidad para formar hifas (Hogan & Kolter, 2004). Sin embargo, también se ha demostrado que la competencia interespecífica repercute en las interacciones sociales intraespecíficas (Harrison *et al*., 2008), lo cual ha generado una nueva visión para el estudio de las interacciones.

3.1 Biofilms mixtos de hongos y bacterias

Los biofilms son agregados de microorganismos unidos por material extracelular constituido por proteínas, lípidos, polisacáridos, ADN, RNA. Generalmente los biofilms se encuentran adheridos a una superficie biótica o abiótica (Davey & O´Toole, 2000). Se sabe que en la naturaleza las bacterias forman comunidades complejas de microorganismos, por lo cual es raro encontrarlas como cultivos plantónicos puros. En ambientes naturales los biofilms mixtos hongo-bacteria participan en diversos procesos, desde la degradación de substratos, hasta el establecimiento de asociaciones sinérgicas complejas para la adquisición de nutrientes.

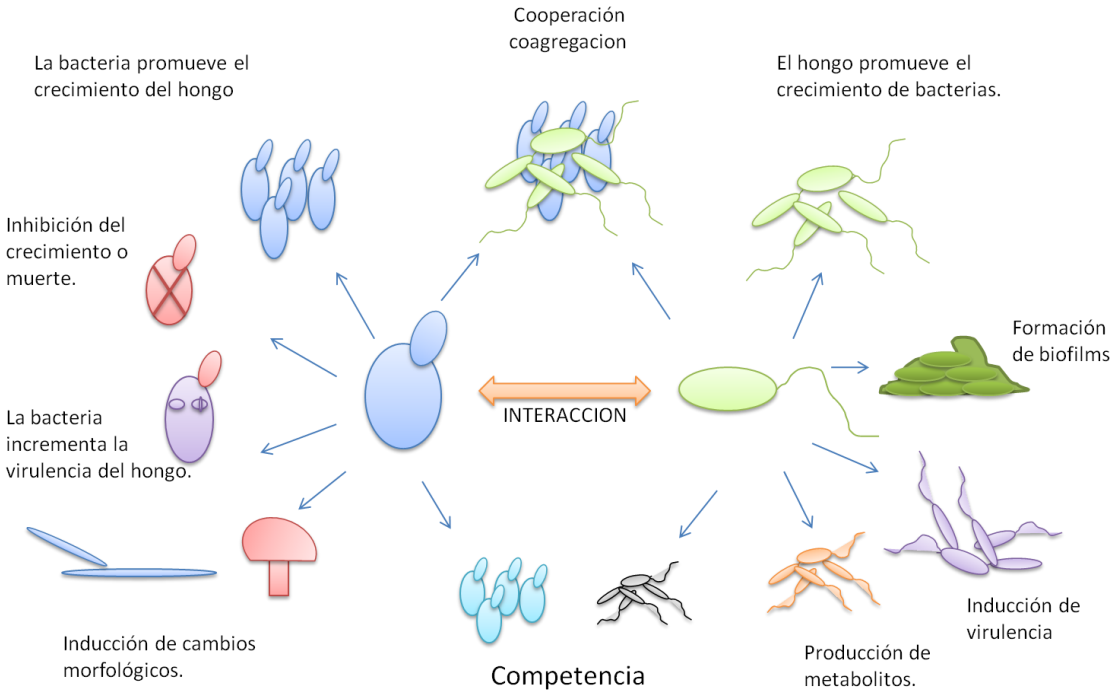

Figura 1.- En este esquema se resumen los eventos que se han identificado en las interacciones entre hongos y bacterias. Estos eventos dependen de las especies involucradas en la interacción. Se pueden distinguir efectos benéficos de una especie sobre la otra para generar cooperación o mutualismo, también puede ocurrir que las especies compitan por nutrientes y se establezca una "guerra química". Es importante destacar que estas interacciones son dependientes de los factores ambientales o del ambiente del hospedero.

Candida albicans causa un gran número de infecciones relacionadas con dispositivos médicos implantados (Thomas *et al.*, 2004). Evidencias obtenidas anteriormente sugieren que estas infecciones asociadas a implantes médicos son el resultado de la formación de biofilms por especies de *Candida* (Kojic & Darouiche, 2004). *C. albicans* y la bacteria *Staphylococcus aureus*, habitan en cavidades del cuerpo y en la piel como organismos comensales, cuando se dan condiciones favorables para el desarrollo de una infección, ambos organismos pueden cambiar de comensales a patógenos y tener efectos adversos sobre el hospedero. *C. albicans* ocasiona candidiasis en la cavidad oral y vaginal, forma biofilms en los catéteres implantados y se disemina en pacientes inmunocomprometidos. Con un comportamiento similar al de *C. albicans*, *S. aureus* se aprovecha del tejido dañado para producir infecciones letales, y se ubica

entre los agentes infecciosos más frecuentes en las infecciones nosocomiales. En general, al perturbar las funciones normales del hospedero se abren las puertas para que estos microorganismos proliferen como patógenos. En el caso de implantes médicos y enfermedades dentales, este par hongo-bacteria colaboran, formando biofilms mixtos (Crump & Collignon, 2000; Wargo & Hogan, 2006). La prevalencia y coocurrencia de *C. albicans* y *S. aureus* sugiere que ambos requieren condiciones similares para ocasionar una infección. Se ha especulado que estas dos especies establecen una cooperación, sin embargo se desconocen los mecanismos involucrados en la interacción.

En el análisis de un gran número de fluidos biológicos de origen humano, Hermann *et al.* (1999) describieron una correlación positiva entre la presencia de *Pseudomonas aeruginosa* y *C. albicans*, indicando que estos dos organismos coexisten dentro del hospedero. En la interacción *C. albicans-P. aeruginosa*, la bacteria forma biofilms sobre las hifas del hongo y lo utiliza como un sustrato potencial y como un origen de alimento. Cuando la bacteria encuentra hifas del hongo se adhiere a la superficie y ocasiona daño celular con una combinación de factores de virulencia (Hogan & Kolter, 2002).

A pesar de que la formación de biofilms mixtos se ha observado en diversos sistemas, los mecanismos moleculares que gobiernan estos procesos no han sido bien entendidos. Estudios enfocados en la interacción entre hongos y bacterias en la fitósfera han mostrado procesos que contribuyen a la formación de biofilms mixtos incluyendo quimiotaxis hacia las células del hongo, el intercambio de nutrientes entre las especies interactuantes, y la expresión de genes específicos al momento de la interacción física. Usando sistemas modelos de biofilms hongo-bacteria, se podrá identificar elementos necesarios para el establecimiento en superficies de plantas o animales.

Los biofilms mixtos compuestos por hongos y bacterias pueden tener propiedades estructurales y fisiológicas que son distintas a las de los biofilms de una sola especie (Wargo & Hogan, 2006), por lo cual se ha especulado que las infecciones mixtas presentan una mayor resistencia a los antibióticos.

3.2 Comunicación celular en las interacciones Hongos-Bacterias

Algunas de las señales químicas que primero fueron identificadas por sus funciones dentro de una especie también son usadas para transmitir información a través de los dominios de la vida. El estudio de la señalización entre dominios se ha enfocado principalmente en las interacciones entre bacterias y eucariotas (Joint *et al.* 2002).

La comunicación célula-célula en bacterias es realizada a través del intercambio de moléculas señales llamadas autoinductores (Figura 2), entre otros. Este proceso, llamado *quorum sensing*, (percepción de quórum) les permite a las bacterias censar la presencia de otras bacterias y responder a las fluctuaciones en número y/o especies presentes por medio de la alteración de comportamientos particulares. La mayoría de los sistemas de *quorum sensing* son específicos para una especie o para un grupo, lo cual presumiblemente previene una confusión en ambientes con diferentes especies. Sin embargo, algunos circuitos de *quorum sensing* controlan el comportamiento que involucra interacciones entre especies bacterianas. Estos circuitos pueden involucrar mecanismos de comunicación intraespecíficos y entre especies. Por otra parte, las estrategias anti- *quorum sensing* están presentes en las bacterias y en los eucariotas, y aparentemente son diseñadas para combatir bacterias que utilizan la comunicación célula–célula para adaptarse a nichos particulares (Taga & Bassler, 2003). Se ha reportado que la macroalga marina *Delisea pulchra* produce furanonas alogenadas, las cuales actúan como moléculas señal antagónicas para la bacteria *Serratia liquefaciens* afectando su circuito de *quorum sensing* e inhibiendo su motilidad. La estrategia anti-*quorum sensing* presentada por el alga marina le da la ventaja de no ser colonizada por la bacteria (Rasmussen *et al.*, 2000). Similarmente, varios organismos producen enzimas que modifican a las AHLs (Acil-homoserinas lactonas) haciéndolas inactivas. Se ha demostrado que enzimas como la lactonasa (AiiA) aislada de especies de *Bacillus* hidrolizan el anillo lactónico de las AHLs para formar acil-homoserina la cual no puede funcionar como una señal de quorum sensing. Aún no se sabe cuál es el papel *in vivo* de AiiA en especies de *Bacillus*, se sugiere que podría ser usado para interferir con comportamientos mediados por quorum sensing de bacterias del suelo que compiten con *Bacillus* por el mismo nicho (Dong *et al*., 2002). Otra enzima inactivadora de AHLs, AiiD, fue recientemente descubierta en una cepa de *Ralstonia* aislada de un biofilm bacteriano. AiiD al igual que AiiA hidroliza AHLs, pero en este caso la cadena acil es cortada quedando intacto el anillo de la homoserina lactona. Se ha demostrado que la introducción de AiiD en *P. aeruginosa*

causa una reducida acumulación extracelular de AHLs inhibiendose comportamientos regulados por AHLs tales como motilidad en swarming, producción de factores de virulencia y la capacidad de la bacteria para causar parálisis de *Caenorhabditis elegans* (Lin *et al*., 2003). En otro estudio, la bacteria *Variovorax paradoxus* demostró ser capaz de usar AHLs como única fuente de carbono y de nitrógeno, sugiriendo que esta bacteria tiene enzimas para degradar AHLs (Leadbetter & Greenberg, 2000).

Estos descubrimientos recientes indican que la inhibición de *quorum sensing* mediante enzimas que degraden las moléculas señales o por medio de moléculas que perturben los circuitos de *quorum sensing*, están ampliamente extendidos en las bacterias y eucariotas. Sin embargo no se conoce ningún caso en el que un hongo inhiba el *quorum sensing* de una bacteria o en el que una bacteria afecte la comunicación intraespecífica en hongos.

Por otra parte, en lugar de la inhibición de la señalización mediada por AHLs, algunas bacterias usan AHLs para la comunicación entre especies durante una infección. Por ejemplo, se ha demostrado que en fibrosis cística los pulmones colonizados por *P. aeruginosa* y *Burkholderia cepacia*; *B. cepacia* responde a AHLs producidas por *P. aeruginosa*, lo cual promueve la formación de un biofilm de especies mixtas (Riedel *et al*., 2001).

Las AHLs tales como la 3-oxo-C12-homoserina lactona producida por *P. aeruginosa* inhiben la ruta Ras1-AMPc-proteina cinasa A (PKA) la cual controla el desarrollo de levadura a hifa en *C. albicans* (Figura 2) (Davis-Hanna *et al*., 2008). Se ha propuesto que la inhibición del desarrollo de las hifas inducido por AHLs contribuye a la coexistencia de ambas especies en infecciones mixtas, ya que las células de levadura muestran mayor sobrevivencia con respecto a las hifas en los cocultivos con *P. aeruginosa*.

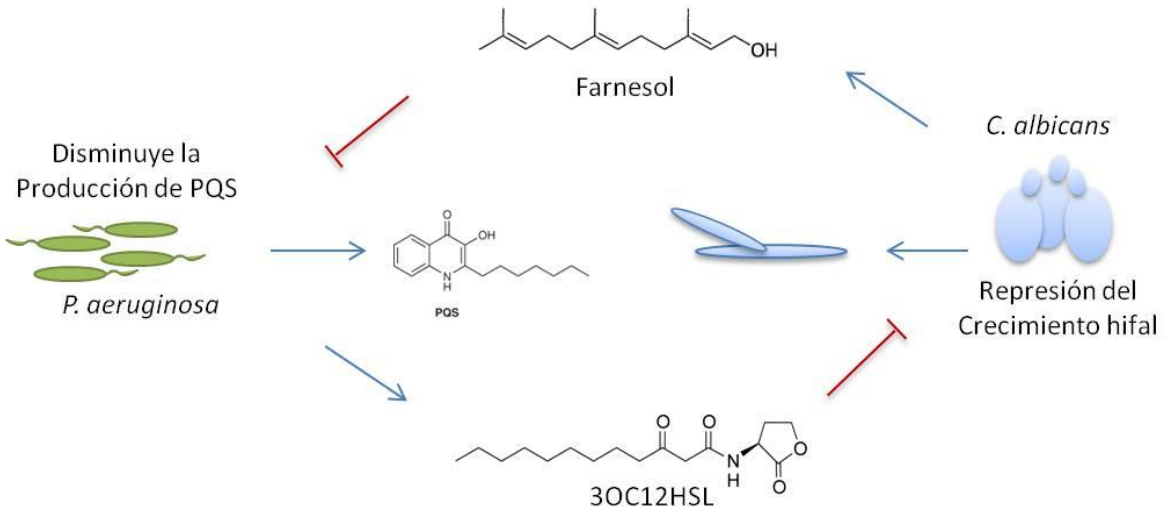

Figura 2. Comunicación cruzada entre *P. aeruginosa* y *C. albicans*. La bacteria produce 3-oxo-C12-homoserina lactona (3OC12HSL) que reprime la formación de hifas en células de *C. albicans*. La bacteria responde al farnesol (una molécula señal producida por el hongo) disminuyendo la producción de PQS (*Pseudomonas* Quinolone Signasl). Modificado de Davis-Hanna *et al*., 2008.

La exploración de sistemas de señalización entre diferentes especies ha mostrado que existe una comunicación cruzada entre especies relacionadas e incluso entre especies de diferentes reinos. Por ejemplo, algunas moléculas señal de bacterias pueden modular el desarrollo de hongos y de otras especies bacterianas. El sesquiterpeno farnesol es una señal intraespecífica para *C. albicans* que inhibe la transición de levadura a hifa (Hogan, 2006), y el acido cis-11-metil-2 dodecenoico denominado factor señal difusible (DSF) de la bacteria *Xanthomonas campestris* controla la formación de biofilm y la expresión de factores de virulencia (Torres *et al*., 2007). DSF puede actuar como una señal entre especies así como, mimetizar el efecto del farnesol en *C. albicans* (Wang *et al*., 2004). Estos resultados soportan el papel potencial que puede tener esta molécula como una señal interespecífica.

El farnesol altera la actividad de un regulador transcripcional en *P. aeruginosa* cuando se aplica como un compuesto purificado o durante un cocultivo con *C. albicans*, disminuyendo la producción de piocianina y de la quinolonas señal de *P. aeruginosa* (PQS) (Cugini *et al*., 2007) y disminuyendo el swarming (McAlester *et al.*,2008). Considerando la habilidad del farnesol y el

DSF para mediar la comunicación entre hongo y bacteria, uno se pregunta si el DSF también tiene un efecto en *P. aeruginosa* (Cugini *et al*., 2007).

Estudios recientes han reportado que los peptidoglicanos actúan como una señal que influyen en el desarrollo de *C. albicans*. Una combinación de química y bioquímica fue usada para identificar fragmentos de peptidoglicanos en suero humano los cuales son poderosos inductores de la formación de hifas en *Candida albicans* (Xu *et al*., 2008). Otro estudio muestra que dipéptidos murámicos pueden entrar a células de hongos y unirse a una adenilato ciclasa especifica que induce la producción de cAMP y crecimiento hifal. Inicialmente este estudio parece involucrar una interacción eucariotica exclusiva, y un aspecto intrigante es que los mamíferos no pueden producir acido murámico, sugiriendo que las altas concentraciones de estos compuestos encontrados en suero humano son originalmente producidas por la microbiota intestinal bacteriana (Xu *et al*., 2008).

Debido a que la mayoría de las bacterias residen en ambientes de especies mixtas, otros ejemplos de conspiraciones bacterianas para realizar diferentes comportamientos podrían ser descubiertos al estudiar el *quorum sensing* en poblaciones mixtas.

3.3 Importancia medica de las interacciones entre hongos y bacterias.

A pesar de que los datos sobre la relevancia clínica de las interacciones hongo-bacteria son limitados, varios estudios han descrito la asociación de bacterias y diferentes especies de *Candida* en un rango de especímenes clínicos (Hermann *et al*., 1999, Adair *et al*.,1999, Klotz *et al*., 2007). Aun no está claro si factores como una terapia sistémica antibacteriana, el estatus del sistema inmune del hospedero o la exposición a patógenos adquiridos en hospitales pueden predisponer a un paciente a la colonización de bacterias y hongos. Sin embargo, las infecciones de especies mixtas pueden tener consecuencias que difieren de aquellas que son asociadas con infecciones ocasionadas por una sola especie. Un estudio de neumonía causada por *Pseudomonas aeruginosa* sugirió que la colonización del tracto respiratorio con especies de *Candida spp.* podría incrementar el riesgo de contraer la enfermedad (Azoulay *et al*., 2006). Otros estudios han mostrado, que individuos con colonización traqueobronquial por especies de

Candida, al ser tratados con antifúngicos tienen un menor riesgo de desarrollar neumonía pseudomonal que aquellos que no fueron tratados con antifúngicos (Nseir *et al*., 2007).

Estudios en humanos sugieren que la mortalidad por infecciones de torrente sanguíneo que son causadas por bacterias o especies de *Candida* están en un rango de 10-40 % (Gudlaugsson *et al*., 2003). Sin embargo se han conducido pocos estudios en donde se realice una comparación de infecciones mixtas contra infecciones de una sola especie. En un estudio se identificó una tasa de sobrevivencia pobre para una infección de torrente sanguíneo ocasionada por especies mixtas de bacterias y especies de *Candida* comparada con una infección ocasionada solo por especies de *Candida* (Dyess *et al*., 1985). Los análisis de las implicaciones de infecciones mixtas son limitados y los ensayos clínicos en humanos en pocas ocasiones son posibles. Ademas, los estudios observacionales son confusos por el hecho de que los pacientes con infecciones polimicrobianas pueden tener otros factores de riesgo que correlacionan con un resultado clínico pobre, tales como una mayor gravedad de la enfermedad o una terapia inadecuada contra uno o ambos microorganismos infecciosos. Además, al describir los mecanismos moleculares por los cuales ocurre cualquier cambio en la virulencia en una infección polimicrobiana es difícil cuando se estudian enfermedades humanas

3.4 Interacción de *Candida albicans* con diferentes especies de bacterias y el impacto de estas asociaciones en su patogenicidad.

Uno de los primeros estudios en donde se prueban las consecuencias patogénicas de una infección mixta hongo-bacteria evaluó la interacción entre *C. albicans* y *Escherichia coli* en ratones (Gale & Sandoval, 1957). Interesantemente, se observó una diminución de la muerte del hospedero ocasionada por *C. albicans* cuando se suministro una dosis subletal de *E. coli* por vía intravenosa o intraperitoneal antes de una dosis letal de *C. albicans*. Experimentos in vitro mostraron que la disminución de la letalidad de *C. abicans* podría deberse a que *E. coli* fue capaz de reducir la viabilidad de *C. albicans* a través del tiempo (Gale & Sandoval, 1957). En contraste, si *E. coli* era inoculada después de *C. albicans*, se observaba un incremento en la letalidad, la cual se pensó que era mediada por la endotoxina de *E. coli* (Gale & Sandoval, 1957). Varias investigaciones han tenido resultados similares, en donde infecciones simultaneas con *C. albicans* y *E. coli* ocasionan mayor mortalidad con respecto a las infecciones generadas por cada uno de los organismos individualmente (Akagawa *et al*., 1995; Klaerner *et al*., 1997), la

endotoxina pareció ser la responsable del incremento de la virulencia de *C. albicans* (Akagawa *et al*., 1995; Burd *et al*., 1992). Estos hallazgos tienen importancia clínica debido a que *E coli* y *C. albicans* son comensales del tracto gastrointestinal humano y a menudo son encontrados en infecciones intraabdominales y en infecciones de torrente sanguíneo adquiridas en hospitales.

Otro ejemplo en donde se incrementa la virulencia de *C. albicans* en presencia de una bacteria es mostrado en un modelo de infección con *Pseudomonales* en heridas causadas por quemaduras (Neely *et al*., 1986). Una candidemia en víctimas humanas con quemaduras es a menudo precedida por una infección bacteriana, especialmente por *P. aeruginosa*. Cuando se pre-infectaron las quemaduras de ratones con una dosis subletal de *P. aeruginosa* y después fueron expuestas a un inoculo subletal de *C. albicans*, los ratones tuvieron una tasa de mortalidad de 60% (Neely *et al*., 1986). En contraste, los ratones quemados que fueron infectados individualmente con cada uno de los microorganismos tuvieron una tasa de mortalidad ≤ 10%. Interesantemente, los estudios de quemaduras y de órganos periféricos en ratones coinfectados mostraron que las muertes parecían deberse a *C. albicans*. Además, los autores encontraron que la enzima proteolítica elastasa B (LasB; también conocida como Pseudolisina) de *Pseudomonas*, fue responsable del incremento de la virulencia de *C. albicans*, pero los detalles de este mecanismo no fueron claros. En un estudio similar usando el modelo de rata, los investigadores encontraron que a las ratas que se les daba una dosis subletal de *P. aeruginosa* desarrollaron neumonía solo en presencia de células viables de *C. albicans* (Roux *et al*., 2009).
En otro estudio donde se ha podido revelar el mecanismo del incremento de la virulencia de *C. albicans* en el entorno de una infección bacteriana, se encontró que las moléculas de péptidoglicano bacteriano encontradas en suero humano, conocidas como dipeptidos de muramil, actúan como un potente inductor para el desarrollo hifal de *C. albicans* (Xu *et al*., 2008). La formación de hifas es un determinante crucial para la virulencia de *C. albicans* en infecciones de mamíferos (Lo *et al*., 1997). Se piensa que los dipéptidos muramicos son originados por las bacterias comensales, y es plausible que las infecciones bacterianas sistemáticas generen una gran cantidad de estas moléculas que promueven el crecimiento hifal que subsecuentemente incrementan la virulencia de *C. albicans.*

3.5 *C. albicans* incrementa la virulencia bacteriana.

Trabajos recientes muestran que los hongos pueden incrementar la virulencia bacteriana, ilustrando un nuevo aspecto de las infecciones polimicrobianas. Esto está bien descrito en estudios en donde se evaluó la virulencia de una infección mixta *C. albicans* y *Staphylococcus aureus* en ratones (Carlson, 1983a; Carlson & Johnson, 1985). Cuando *S. aureus* fue inoculada intraperitonealmente, no se detectaron células bacterianas en la sangre, páncreas o en bazo y los ratones sobrevivieron. Sin embargo, cuando la misma dosis de *S. aureus* fue administrada con una dosis subletal de *C. albicans* 48 horas más tarde se pudo detectar células de la bacteria y del hongo en todas las muestras de sangre y órganos abdominales y una alta mortalidad (Carlson & Johnson, 1985). Por otro lado, no se observaron diferencias en la carga fúngica de los órganos infectados solo con *C. albicans* o coinoculados con *S. aureus*. Interesantemente la histopatología de la cavidad peritoneal indico que *S. aureus* siempre fue encontrada en sitios en donde había crecimiento hifal, aun cuando los dos patógenos fueron inyectados en diferentes sitios. Además, en el contexto de una infección mixta, en los órganos peritoneales se desarrollaron altas cargas de *S. aureus* pero cantidades no significativas de células de *C. albicans (*Carlson & Johnson, 1985). Los autores proponen que *C. albicans* proporciona protección para *S. aureus* en la cavidad peritoneal y mejora su virulencia permitiendo que la bacteria se disemine hacia los tejido peritoneales. Sin embargo, los efectos observados pueden depender de la cepa bacteriana (Carlson, 1983a). Hallazgos similares fueron observados por el mismo investigador en las bacterias Gram negativa *Serratia marcescens* y en *Enterococcus faecalis* (Carlson, 1983b), las cuales son importantes patógenos de humanos que causan infecciones nosocomiales que a menudo se originan de una fuente intraabdominal. Dada la importancia de *S. aureus* como un patógeno de humano, y su habilidad para causar infecciones que se diseminan y su establecimiento común con *C. albicans*, estos hallazgos son de gran importancia para el entendimiento de la patogénesis de *S. aureus* en humanos. Además, estos datos también sugieren un beneficio indirecto de un tratamiento antifúngico en el contexto de una infección compleja hongo-bacteria, el cual generaría una disminución de la virulencia bacteriana.

3.6 Antagonismo entre *Candida* y *Pseudomonas*.

Se ha demostrado que la producción de fenazinas por *Pseudomonales* es de gran importancia para las interacciones con hongos (Thomashow & Weller, 1988; Anjaiah *et al*., 1998; Bolwerk *et al*., 2003). Por ejemplo, *Pseudomonas chlororaphis* coloniza y forma biofilms sobre el

fitopatógeno *Fusarium oxysporium* y contribuye al biocontrol del hongo por medio de la secreción de fenazina-1-carboxamida (Bolwerk *et al*., 2003). *P. aeruginosa* secreta dos fenazinas adicionales, fenazina-1-carboxylato (PCA) y piocianina (PYO), las cuales también se ha demostrado que antagonizan el crecimiento del hongo (Thomashow *et al*., 1990; Kerr *et al*., 1999). Recientemente se demostró que en cocultivos de *P. aeruginosa –C. albicans* , *P. aeruginosa* produce 5 metil-fenazinio-1- carboxilato (5MPCA). 5MPCA es más eficiente en inducir la muerte del hongo que las fenazinas PCA o PYO (Gibson *et al*., 2009). Mientras que 5MPCA ha sido previamente descrita como un supuesto precursor de PYO, un papel biológico para esta fenazina ha sido descrito pobremente porque esta molécula no se acumula en el medio de monocultivos de *P. aeruginosa* (Byng *et al*., 1979). Otro trabajo demuestra que *C. albicans* consume 5MPCA Y PMS a través del tiempo, y que la actividad metabólica y condiciones reductoras dentro de colonias o de biofilms facilitan la acumulación. PMS y 5MPCA fueron concentradas dentro de las células fúngicas y la acumulación de esta fenazina se debe a que se une covalente a los grupos aminos de las biomoléculas del hongo (Morales *et al*., 2010). Estos autores proponen que la localización permanente de estos derivados de metilfenazinio dentro de las células permite una constante generación de niveles tóxicos de especies reactivas de oxigeno, y junto con la modificación de macromoléculas, son la causa de la muerte del hongo.

3.7 Sideróforos en las interacciones hongo-bacterias.

La disponibilidad de hierro es un factor importante para el crecimiento de los microorganismos en diferentes ambientes. En mamíferos, las proteínas lactoferrina y transferrina secuestran el hierro, y los patógenos o comensales crecen solo si tienen la capacidad de adquirir el hierro unido a estas proteínas del hospedero. Una estrategia frecuente usada por hongos y bacterias para la adquisición de hierro es la secreción de moléculas quelantes denominadas sideróforos (Figura 2), las cuales pueden tomar el hierro unido a las proteínas del hospedero debido a su alta afinidad por el metal (Howard, 1999; Ratledge & Dover, 2000). El complejo sideróforos-hierro es tomado por sistemas de transportadores específicos; sin embargo, los microorganismos tienen sistemas transportadores para utilizar sideróforos heterólogos producidos por otras especies. Por ejemplo, *Escherichia coli* no solo posee transportadores para homólogos de enterobactina y aerobactina sino también para sideróforos de tipo hidroxamato producidos por hongos (Ratledge & Dover, 2000). El cuerpo humano contrarresta las estrategias de microorganismos patógenos, por

ejemplo, reduciendo el contenido de hierro libre en los fluidos utilizando proteínas tales como las transferrinas (Weinberg, 1999).

P. aeruginosa produce dos sideróforos, pioverdina y pioquelina, los cuales son elementos importantes en la interacción con el hospedero y en la competencia por hierro entre microbios (Figura 3).

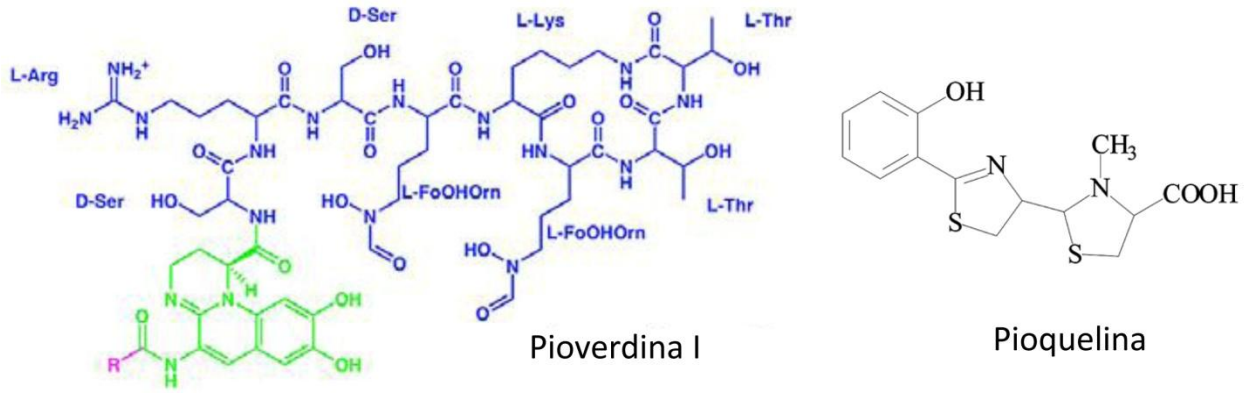

Figura 3. Estructura molecular de los principales sideróforos producidos por *P. aeruginosa*.

La producción y utilización de sideróforos se ha observado en diferentes especies de hongos (Howard, 1999). Sin embargo, la levadura *Saccharomyces cerevisiae* no produce sideróforos, pero es capaz de tomar hierro por un sistema de reducción, el cual incluye las proteínas Fre1p y Fre2p (Askwith *et al*., 1996). Como alternativa al sistema de reducción de hierro, *S. cerevisiae* presenta un sistema para utilizar sideróforos producidos por otros hongos o bacterias (Lesuisse *et al*., 2001). En esta levadura las proteínas Fit1p, Fit2p y Fit3p son requeridas para la toma de los sideróforos Ferrioxamina B y ferricromo pero no para triacetilfusarinina y enterobactina (Protchenko *et al*., 2001).

Algunos reportes han descrito la producción de sideróforos de tipo hidroxamato por *C. albicans*; sin embargo, la estructura de tales moléculas no a sido elucidada (Ismail, 1985; Sweet & Douglas, 1991). *C. albicans* al igual que *S. cerevisiae* también tiene capacidad de utilizar sideróforos producidos por bacterias (enterobactinas) u hongos (ferricromo) (Minnick *et al*., 1991).

Hasta el momento se desconoce el papel que juegan los sideróforos en las interacciones que ocurren en las infecciones polimicrobianas, y por lo tanto no se conocen sus funciones en el establecimiento y desarrollo de las interacciones hospedero-patógenos.

4. PLANTEAMIENTO DEL PROBLEMA.

Varios estudios indican que en las interacciones entre microorganismos, las especies expresan fenotipos y comportamientos que difieren de aquellos observados en cultivos puros. Por ejemplo, se sabe que las especies involucradas en infecciones polimicrobianas son más virulentas y muestran una mayor resistencia a los agentes antimicrobianos, lo cual resulta en un incremento en la persistencia de la infección. Aquí nosotros analizamos la interacción entre dos patógenos oportunistas, *C. albicans* y *P. aeruginosa*, para conocer las causas del incremento en la virulencia y persistencia de los biofilms mixtos.

5. OBJETIVO GENERAL

Conocer los mecanismos moleculares involucrados en la interacción *C. albicans-P. aeruginosa* y sus efectos en la virulencia de las especies.

6. OBJETIVOS PARTICULARES.

Conocer los factores ambientales que influyen en la naturaleza de la interacción entre *C. albicans* y *P. aeruginosa*.

Identificar los cambios en el proteoma de *C. albicans* y *P. aeruginosa* generados por la interacción interespecífica.

Identificar las relaciones entre los cambios en el proteoma y el desarrollo de la interacción.

Conocer el efecto de la interacción en la patogenicidad de las especies.

7. ESTRATEGIA EXPERIMENTAL

Se realizo un análisis de microscopía láser confocal (MLC) para identificar las etapas de desarrollo de los biofilms mixtos y monoespecíficos. Se analizó la viabilidad de las células para identificar cambios en la naturaleza de la interacción

Los biofilms mixtos y monoespecíficos fueron cultivados en un medio sintético completo (RPMI), en condiciones hipóxicas en cajas Petri de poliestireno. Después de 24 horas de cultivo, las células no adheridas fueron removidas y el biofilm fue despegado del fondo de las cajas. Las células de la bacteria y del hongo fueron separadas de los biofilms mixtos por centrifugación en un gradiente de Percoll. Las células de biofilms monoespecíficos también fueron sometidas al proceso de centrifugación. Se realizo extracción de proteínas totales y se analizaron mediante electroforesis en dos dimensiones. Se realizo la comparación de los perfiles de expresión entre células de biofilms mixtos contra células de biofilms monoespecíficos. Las proteínas diferencialmente expresadas fueron identificadas mediante espectrometría de masas tipo MALDI-TOF. Se realizaron experimentos puntuales para poner a prueba las hipótesis derivadas del análisis de las proteínas diferencialmente expresadas. (Para más detalles de la estrategia experimental consultar materiales y métodos de la publicación anexa).

8. RESULTADOS

8.1 Estructura y desarrollo de los biofilms.

Con el objetivo de identificar las diferentes etapas del desarrollo de los biofilms, se realizó microscopia laser Confocal de biofilms mixtos y monoespecíficos en hipoxia y normoxia. Para obtener las imágenes de fluorescencia, se utilizo el Kit Live/dead de Molecular Probes L7007 (ver detalles en materiales y métodos). Los componentes del kit son: SYTO 9 y Ioduro de propidio. Ambas moléculas se unen a ácidos nucleícos y emiten fluorescencia al ser excitados con la longitud de onda adecuada. El SYTO 9 penetra a células vivas y muertas, por el contrario el ioduro de propidio solo puede entrar a células que manifiestan daños en la membrana celular. Las células con la membrana integra emiten luz verde, mientras que las células dañadas emiten luz roja, esto debido a que el ioduro de propidio al estar en mayor concentración afecta la

fluorescencia de SYTO 9. Uno de los problemas de este ensayo, es que en determinadas condiciones las células pueden manifestar daños en la membrana (aparecen como muertas) y sin embargo su viabilidad no se ve afectada (datos del fabricante) y son capaces de duplicarse. Las imágenes se obtuvieron en la unidad de servicios de microscopia del Instituto de Biotecnología (UNAM). Se tomaron 4 imágenes de diferentes campos de cada condición.

Realizamos microscopía láser confocal de biofilms mixtos y monoespecíficos a las 5, 10, 24 y 72 h. las imágenes de 5 y 10 h muestran biofilms en desarrollo. A las 24 h toda la superficie esta colonizada por ambas especies. Basados en el análisis de las imágenes proponemos que a 24 h las células están formando un biofilm mixto maduro (Fig. 4). En contraste, a las 72 h se puede apreciar un cambio significativo en la estructura del biofilm mixto, caracterizado por la una reducción en el tamaño de la población de *C. albicans* (Fig. 4). Esto indica que el biofilm está en una etapa de disgregación. La Figura 5 muestra el desarrollo del biofilm de *C. albicans*, el cual a 24 h presenta un comportamiento similar al del biofilm mixto. Sin embargo, a 72 h el número de células viables y de hifas es significativamente mayor con respecto a los biofilms mixtos. Considerando estos resultados, sugerimos que en los biofilms mixtos se establece una antagonismo en el cual la bacteria afecta la viabilidad del hongo.

Los biofilms monoespecíficos de *P. aeruginosa* muestran un desarrollo en el que se puede distinguir una etapa de formación del biofilm (5-10 h), seguida por la formación de un biofilm estructurado (24 h) y una etapa de disgregación a las 72 h (Fig. 6).

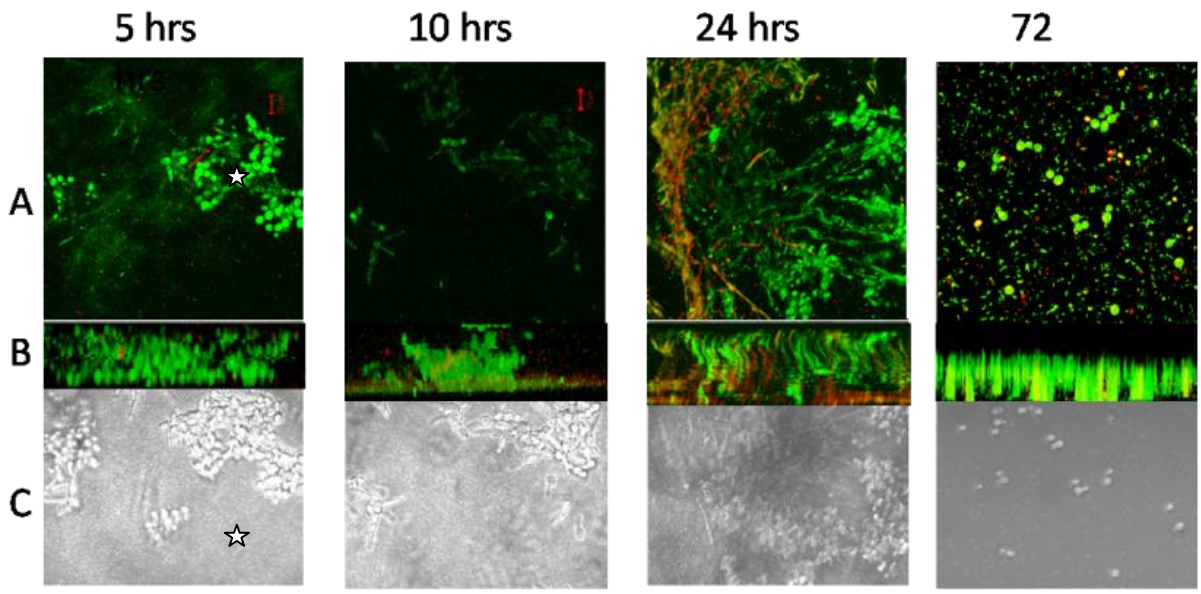

Figura 4. Imágenes de microscopía láser confocal (MLC) de biofilms mixtos *C. albicans-P. aeruginosa* formados en poliestireno en hipoxia a diferentes tiempos. A) imágenes de fluorescencia. B) Vista del grosor de los biofilms. C) contraste de fase. Magnificación 63X.

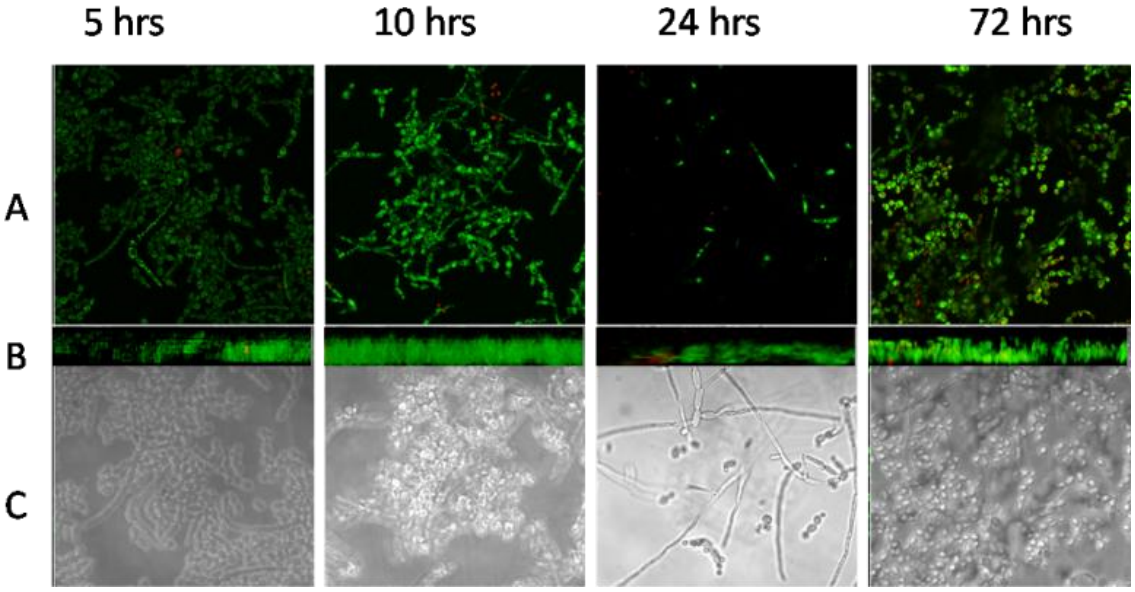

Figura 5. Imágenes de MLC de biofilms de *C. albicans* formados en poliestireno en hipoxia a diferentes tiempos. A) imágenes de fluorescencia. B) Vista del grosor de los biofilms. C) contraste de fase. Magnificación 63X.

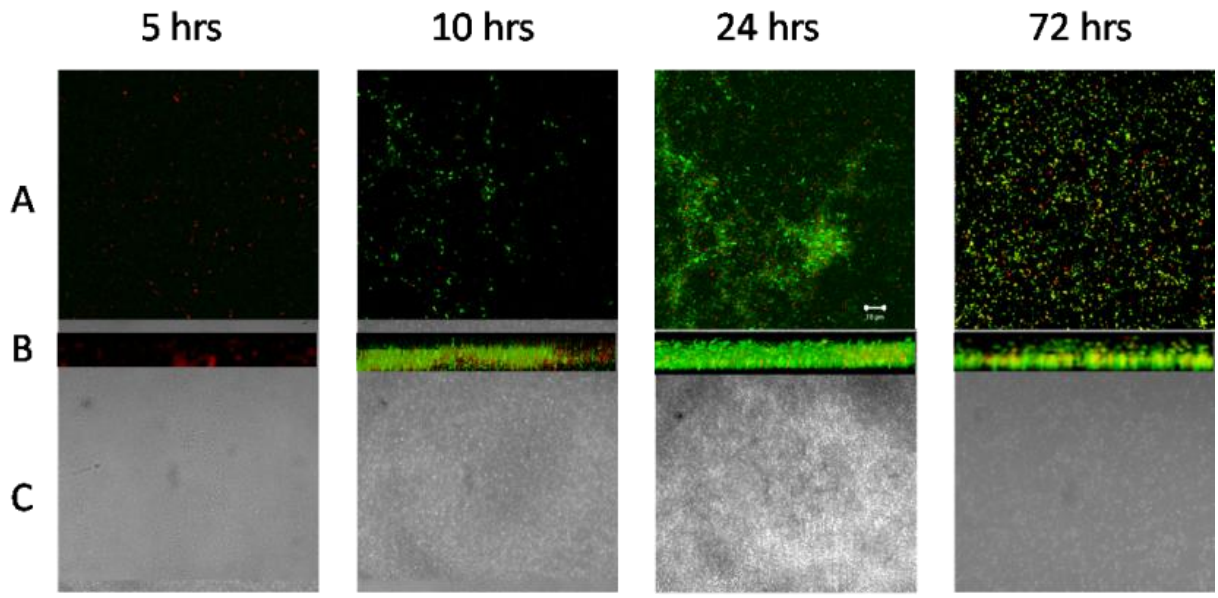

Figura 6.- Imágenes de MLC de biofilms de *P. aeruginosa* formados en poliestireno en hipoxia a diferentes tiempos. A) imágenes de fluorescencia. B) Vista del grosor de los biofilms. C) contraste de fase. Magnificación 63X.

8.2 Análisis proteómico de *P. aeruginosa* y *C. albicans* en biofilms mixtos.

El análisis proteómico de la interacción se realizó a las 24 h de cultivo, considerando que ha este tiempo el hongo y la bacteria forman un biofilm maduro. Los resultados del análisis proteómico se resumen en un manuscrito que fue publicado en *The ISME Journal*.

8.3 Interspecies competition triggers virulence and mutability in *Candida albicans-Pseudomonas aeruginosa* mixed biofilms (artículo publicado).

El 80% de los resultados derivados del presente proyecto fueron integrados en el siguiente manuscrito:

Trejo-Hernández A, Andrade-Domínguez A, Hernández M, Encarnación S. (2014). Interspecies competition triggers virulence and mutability in *Candida albicans-Pseudomonas aeruginosa* mixed biofilms. *The ISME journal* 1–15. doi:10.1038/ismej.2014.53 http://www.nature.com/ismej/journal/vaop/ncurrent/full/ismej201453a.html

The ISME Journal (2014), 1–15

www.nature.com/ismej

ORIGINAL ARTICLE

Interspecies competition triggers virulence and mutability in *Candida albicans–Pseudomonas aeruginosa* mixed biofilms

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Inter-kingdom and interspecies interactions are ubiquitous in nature and are important for the survival of species and ecological balance. The investigation of microbe-microbe interactions is essential for understanding the *in vivo* activities of commensal and pathogenic microorganisms. *Candida albicans*, a polymorphic fungus, and *Pseudomonas aeruginosa*, a Gram-negative bacterium, are two opportunistic pathogens that interact in various polymicrobial infections in humans. To determine how *P. aeruginosa* affects the physiology of *C. albicans* and *vice versa*, we compared the proteomes of each species in mixed biofilms versus single-species biofilms. In addition, extracellular proteins were analyzed. We observed that, in mixed biofilms, both species showed differential expression of virulence proteins, multidrug resistance-associated proteins, proteases and cell defense, stress and iron-regulated proteins. Furthermore, in mixed biofilms, both species displayed an increase in mutability compared with monospecific biofilms. This characteristic was correlated with the downregulation of enzymes conferring protection against DNA oxidation. In mixed biofilms, *P. aeruginosa* regulates its production of various molecules involved in quorum sensing and induces the production of virulence factors (pyoverdine, rhamnolipids and pyocyanin), which are major contributors to the ability of this bacterium to cause disease. Overall, our results indicate that interspecies competition between these opportunistic pathogens enhances the production of virulence factors and increases mutability and thus can alter the course of host-pathogen interactions in polymicrobial infections.
The ISME Journal advance online publication, 17 April 2014; doi:10.1038/ismej.2014.53
Subject Category: Microbe-microbe and microbe-host interactions
Keywords: species interactions; mixed biofilm; virulence; antagonism; siderophores; iron

Introduction

In many natural environments and chronic human infections, microbes live in matrix-encased groups known as biofilms, rather than as free-living (planktonic) cells. In biofilms, cells are known to show distinct expression profiles that are not apparent in planktonic cells. For example, biofilm bacteria are more resistant to killing by antibiotics, biocides and host defenses than planktonic cells and may be less vulnerable to predators (Peleg *et al.*, 2010). Microbial biofilms represent heterogeneous populations of species that form intimate contacts. Within these populations, species communicate, cooperate and compete (Davey and O'toole, 2000; Tyson *et al.*, 2004; Hansen *et al.*, 2007; Harrison *et al.*, 2008; Garbeva *et al.*, 2011). Some of these microbial interactions are beneficial, and some are detrimental from a human standpoint (Hughes and Kim, 1973; Lynch and Robertson, 2008).

The interactions between *Pseudomonas aeruginosa* and *Candida albicans* provide a model for many bacteria–eukaryote interactions. An in-depth understanding of these interactions could be exploited for the benefit of mankind. *Pseudomonas* and *Candida* are organisms that are commonly isolated from the sputum of cystic fibrosis patients (Hughes and Kim, 1973; Bauernfeind *et al.*, 1987; Bakare *et al.*, 2003). Both species are common non-pathogenic commensals of healthy individuals. In compromised individuals, they are able to initiate invasive growth that may result in serious disease and death (Naglik *et al.*, 2004; Hube, 2006; Pfaller and Diekema, 2007). *C. albicans* is a dimorphic fungus that can live as a yeast or in a filamentous form. In response to certain stimuli (signaling molecules, temperature and host factors), it switches from its yeast to its hyphal form. Both morphological forms are important for

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virulence, and the ability to undergo morphological transformation is therefore an important virulence trait (Gow, 1997; Calderone and Fonzi, 2001; Gow *et al.*, 2002; Liu, 2002; Whiteway and Oberholzer, 2004). Several studies have suggested that *P. aeruginosa* may inhibit *C. albicans* growth within the host (Bauernfeind *et al.*, 1987; Kerr, 1994; Burns *et al.*, 1999; Gupta *et al.*, 2005; Kaleli *et al.*, 2007). Hogan and Kolter (2002) demonstrated that *P. aeruginosa* is cytotoxic to the filamentous form of *C. albicans* but is unable to attach to or kill *C. albicans* yeast cells. In addition, it has been reported that 3-oxo-C12HSL, a signaling molecule produced by *P. aeruginosa*, and structurally related molecules can inhibit and even reverse the switch from yeast to hyphal growth in *C. albicans* (Hogan *et al.*, 2004). Interestingly, *in vitro* studies have demonstrated that these signaling interactions between *P. aeruginosa* and *C. albicans* may in fact be bidirectional. It has been observed that the addition of farnesol leads to decreased *Pseudomonas* quinolone signal (PQS) production in *P. aeruginosa* and reduced levels of the PQS-regulated virulence factor pyocyanin (Cugini *et al.*, 2007).

In a burned mouse model, Neely *et al.* (1986) demonstrated that *Pseudomonas* infections could predispose burned mice to fatal candidiasis and that the proteolytic activity generated by the bacteria was primarily responsible for the establishment of lethal fungal infections.

To understand the interactions between pathogens and the resident microbiome and how they affect gene expression patterns in the pathogens and contribute to bacterial diseases, Duan *et al.* (2003) investigated the interactions between pathogenic *P. aeruginosa* and avirulent oropharyngeal flora strains isolated from sputum samples of cystic fibrosis patients. Their results suggest that important contributions of the host microflora to *P. aeruginosa* infection occur via the modulation of gene expression through interspecies communications (autoinducer-2-mediated quorum sensing).

Using a *Drosophila* model of polymicrobial infections, Sibley *et al.* (2008) demonstrated that a large proportion of the organisms found in cystic fibrosis airways have the ability to act synergistically with *P. aeruginosa*. This synergy involves microbe–microbe interactions that result in modulation of *P. aeruginosa* virulence factor gene expression within infected *Drosophila*. Nevertheless, the mechanisms underlying this synergy were not identified.

Few studies have demonstrated that physiological processes are involved in the *C. albicans–P. aeruginosa* interaction and the impacts of this interaction on the ecology of the *C. albicans–P. aeruginosa* community. To determine how *P. aeruginosa* affects the physiology of *C. albicans* and *vice versa*, we compared the proteome of each species in mixed biofilms with single-species biofilms (Figure 1). In addition, we conducted an analysis of secreted proteins and small molecules involved in the interaction. To our knowledge, this is the first whole-cell proteome analysis of microbial species that coexist and form mixed biofilms.

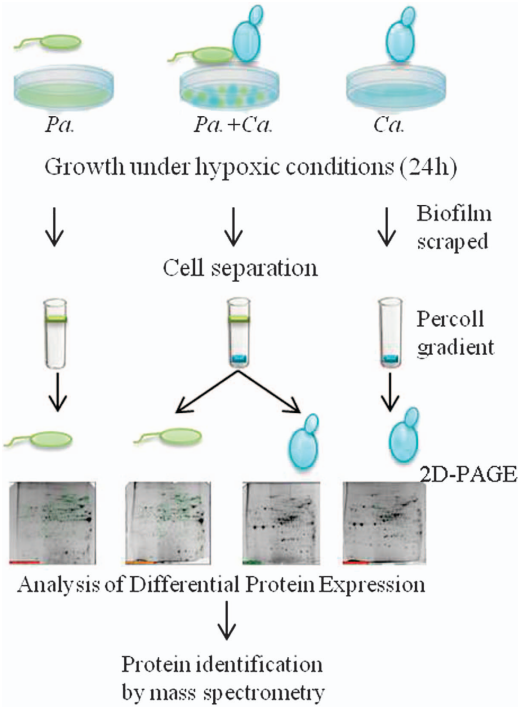

Figure 1 Experimental design for the proteomic analysis of *C. albicans-P. aeruginosa* interaction. Single- and mixed-species biofilms were cultured under hypoxic conditions in polystyrene Petri plates. To generate mixed biofilms, we inoculated with *P. aeruginosa* (Pa.) $\sim 1.5 \times 10^8$ cells ml^{-1} and $\sim 1 \times 10^6$ *C. albicans* (Ca.) cells ml^{-1} in Petri plates at the beginning of each experiment. For the single-species biofilms, the medium initially contained $\sim 1.5 \times 10^8$ bacterial cells ml^{-1} or $\sim 1 \times 10^6$ yeast cells ml^{-1}. At 24 h postculture, the medium with planktonic cells was removed, and the biofilm at the bottom of the plate was scraped. The fungi and bacteria were separated from the mixed biofilms by centrifugation in a discontinuous Percoll gradient. The monospecific biofilms received the same treatment as the mixed biofilms. Whole-cell proteins of three independent cultures (biological replicates) were analyzed by 2D-PAGE. Spots whose relative expression changed in *P. aeruginosa* or *C. albicans* in single versus mixed biofilms were identified by MALDI-TOF mass spectrometry.

Here we show that interspecies competition for iron between these opportunistic pathogens causes encompasses changes in the expression/production of factors that have been previously identified as virulence factors in studies of disease. In consequence, iron competition has the potential to cause significant 'collateral damage' to the host and determines the course and severity of a mixed infection.

Materials and methods

Microbial culture and growing conditions

P. aeruginosa PAO1 (Holloway, 1955) and *C. albicans* CAI4 (*ura3::imm434/ura3::imm434*) (Fonzi and Irwin, 1993) were cultured for 14 h at 30 °C in LB medium or YPD (1% yeast extract, 2% bactopeptone

and 2% dextrose), respectively. The cells were then collected via centrifugation and washed twice with water. These cells were used for interaction experiments. Single- and mixed-species biofilms were cultured in 10-cm diameter polystyrene Petri plates containing 20 ml of RPMI 1640 medium plus L-glutamine (GIBCO no.11875, Grand Island, NY, USA), supplemented with 50 mM dextrose and 40 μg ml^{-1} uridine. This is a complete synthetic medium that contains vitamins, amino acids, inorganic salts and glutathione. It contains no proteins or growth-promoting agents. To generate mixed biofilms, we inoculated with $\sim 1.5 \times 10^8$ bacterial cells ml^{-1} and $\sim 1 \times 10^6$ fungal cells ml^{-1} in each Petri plates at the beginning of an experiment. For the single-species biofilms, the medium initially contained $\sim 1.5 \times 10^8$ bacterial cells ml^{-1} or $\sim 1 \times 10^6$ yeast cells ml^{-1}. The cultures were incubated at 37 °C without shaking.

To obtain biofilms under hypoxic conditions, the plates were wrapped with Parafilm. In contrast, to obtain biofilms under normoxic conditions, the plates were not wrapped. At 24 h postculture, the medium with planktonic cells was removed, and 5 ml of cold stop buffer was added (20 mM sodium azide, 200 mM Tris-HCl pH 8, 20 mM EDTA, 300 μg ml^{-1} rifampicin and 500 μg ml^{-1} chloramphenicol), after which the biofilm that had formed at the bottom of the plate was scraped. The biofilms were collected via centrifugation at 10 000 *g* for 5 min at 4 °C, and the pellet was frozen with liquid nitrogen and stored at − 70 °C.

Determination of populations dynamics
The total population (including planktonic and sessile cells) of each species in the cultures was determined by calculating the number of CFU (colony-forming units) ml^{-1}. Samples of the cultures were diluted in Tween solution (0.01% Tween 80 and 10 mM $MgSO_4$), sonicated for 30 s and plated on LB agar to determine the population densities of *P. aeruginosa*. To estimate the *C. albicans* population density, samples were centrifuged (4 min at 3000 *g*), washed and deflocculated using 300 mM EDTA before being diluted, sonicated for 30 s and plated on YPD agar, as previously described by Smukalla *et al.* (2008).

Iron and oxygen quantitation
The concentration of iron ions in the culture supernatant was determined separately for Fe^{2+} and Fe^{3+} through spectrophotometric assays using iron test kit no. 1.14761.0002 (Merck KGaA, Darmstadt, Germany). The concentration of dissolved oxygen in the culture at 24 h was determined using an OXEL-1 oxygen electrode (World Precision Instruments, Sarasota, FL, USA).

Purification of whole-cell proteins
Whole-cell proteins were isolated from three independent cultures (biological replicates). The fungi and bacteria were separated from the mixed biofilms via centrifugation in a discontinuous Percoll gradient, as described in the Supplementary Methods. The monospecific biofilms received the same treatment as the mixed biofilms. Protein isolation was performed through phenol extraction, as described in the Supplementary Methods.

Extracellular protein extraction
At 24 h after the initiation of the biofilm cultures, the medium was removed and centrifuged at 6000 *g* for 5 min. The supernatant was passed through a 0.45-μm pore-size membrane and stored at − 20 °C. A 400-ml aliquot of the supernatant was subsequently lyophilized, and protein isolation was performed through phenol extraction (Hurkman and Tanaka, 1986).

Two-dimensional gel electrophoresis
The methods applied for sample preparation, preparative two-dimensional gel electrophoresis (2D PAGE) and image analysis have been previously described (Encarnación *et al*., 2003). Through isoelectric focusing, 600 μg of whole-cell protein and secreted protein was separated across a linear pH range of 3–10. All gel experiments were performed with proteins isolated from three independent cultures.

Analysis of differential protein expression
For this analysis, 2D images were captured by scanning Coomassie blue-stained gels using a GS-800 imaging densitometer (Bio-Rad, Hercules, CA, USA) and analyzed with the PDQuest 2-D Analysis software (Bio-Rad). Three gels obtained from three different assays were analyzed to guarantee representative results. Spot normalization, as a means of internal calibration to ensure the independence of the data from experimental variations between gels, was performed using relative volumes (%Vol) to quantify and compare the obtained gel spots. The %Vol corresponds to the volume of each spot divided by the total volume of all of the spots in the gel. Student's *t*-test was performed to assess the significance of differences between differentially expressed proteins. Based on the average spot volume ratio, spots whose relative expression changed at least 1.5-fold (increase or decrease) in *P. aeruginosa* or *C. albicans* in single versus mixed biofilms were considered significantly different, with $P < 0.05$.

Determination of P. aeruginosa *mutability*
The mutability of *P. aeruginosa* in monocultures and cocultures was estimated by determining the frequency of spontaneous mutants resistant to rifampicin (Rif^R). Mutability was evaluated in heat-inactivated fetal bovine serum and RPMI medium under hypoxic conditions. Cultures in RPMI

were performed in Petri plates as described above. Cultures in fetal bovine serum (Lonza, Cat. no. 14-502, Walkersville, MD, USA) supplemented with 10 mM dextrose and 20 μg ml^{-1} uridine were performed in 24-well plates containing 1 ml of fetal bovine serum per well. To generate monocultures, each well was inoculated with $\sim 5 \times 10^6$ bacterial cells ml^{-1}. For the cocultures, the medium initially contained $\sim 5 \times 10^6$ bacterial cells ml^{-1} and $\sim 1 \times 10^7$ *C. albicans* cells ml^{-1}. Plates were wrapped with Parafilm and incubated at 37 °C without shaking for 24 h. Three mililiters of culture were centrifuged, and the cells were spread on a LB plates containing rifampicin (100 μg ml^{-1}) and incubated at 37 °C. Dilutions were also plated on LB plates without antibiotics to determine the total number of CFUs. The colonies were scored for Rif resistance 48 h after plating. The mutation frequencies were approximated as the mean number of Rif^R cells divided by the total number of CFUs.

Statistical analysis

All data were calculated as the mean ± s.d. of at least three experiments. Statistical analysis was performed using the Student's *t*-test, and differences were considered statistically significant at $P < 0 \cdot 05$ compared with control experiments.

Results and discussion

Population dynamics during P. aeruginosa–C. albicans *interaction*

Studies have established that *P. aeruginosa* affects the growth of *C. albicans* in nutrient-rich environments and under normoxic conditions (Hogan and Kolter 2002; El-Azizi *et al.*, 2004). Here we analyzed the development of the *P. aeruginosa–C. albicans* interaction in complete synthetic defined medium under environmental conditions similar to those observed in infections in which both species interact: hypoxic conditions (0.1% dissolved oxygen ± 0.08 s.d.) with low-iron concentrations (67 nM ± 12.5 s.d.). We observed that during the first 24 h the growth dynamics of *C. albicans* and *P. aeruginosa* were similar between cocultures and monocultures. In contrast, 24 h later, the presence of bacteria led to a rapid decrease in the fungal population (Figure 2). Microscopic analysis revealed that, after 48 h of coculture, the fungus

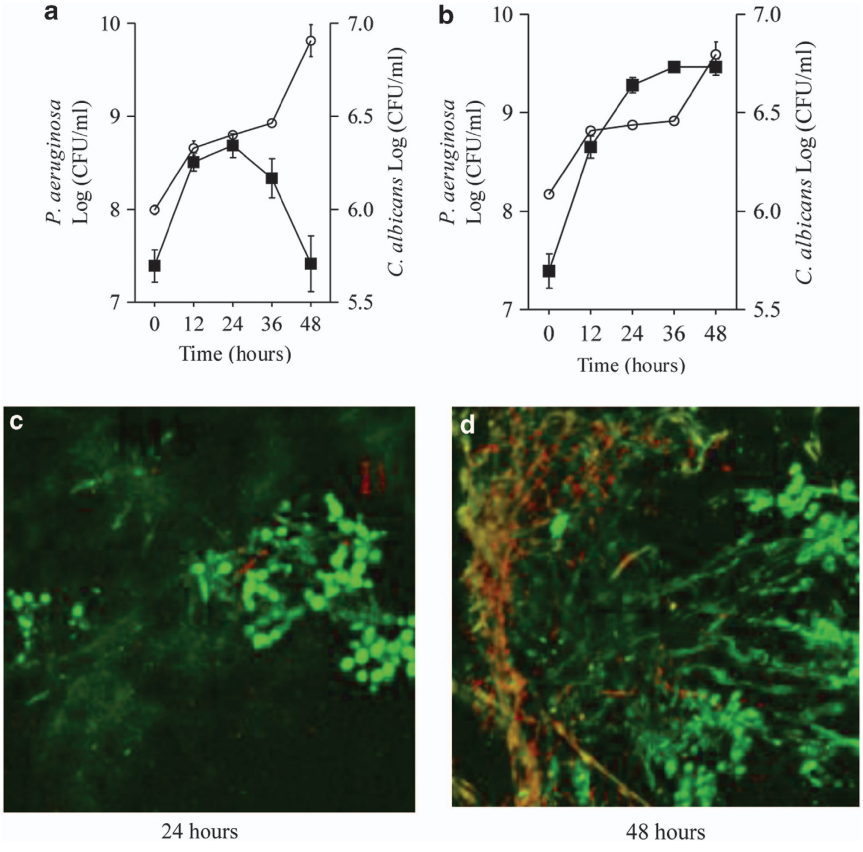

Figure 2 Growth dynamics of *C. albicans* (squares) and *P. aeruginose* (circles) in (**a**) cocultures and (**b**) monocultures. Growth was based on colony-forming units (CFU). (**c**, **d**) Epifluorescence images from mixed *C. albicans–P. aeruginosa* biofilms stained with the LIVE/DEAD staining system are shown. A viability assay confirmed that *C. albicans* cells lost viability after 48 h in mixed biofilms. Red cells (stained with propidium iodide) are considered dead, while live cells remained green (stained with SYTO9). The panels present the means ± s.e.m. ($n = 3$).

cells had been killed by the bacterium (Figures 2c and d).

Microbes competing with unrelated or distantly related species for limited resources in the same niche activate mechanisms such as secretion of proteins or small molecules to attack the competing strains or species (Czaran *et al.*, 2002; Be'er *et al.*, 2009). We hypothesized that during the first 24 h of coculture, the *C. albicans* and *P. aeruginosa* compete for nutrients, after which a parasitism is established in which the bacterium is the parasite, and the fungus is the host.

To understand the molecular mechanisms involved in this interaction, we compared the proteome of each species in mixed biofilms with single-species biofilms after 24 h of coculture.

Low oxygen levels influence the C. albicans–P. aeruginosa *interaction*

Previous *in vitro* studies have demonstrated that 3-oxo-C12 homoserine lactone, a cell–cell signaling molecule produced by *P. aeruginosa*, inhibits *C. albicans* filamentation, without affecting fungal growth rates in cocultures (Hogan *et al.*, 2004). Interestingly, we observed that in cocultures under hypoxic conditions (0.1% dissolved oxygen ± 0.08 s.d.), *P. aeruginosa* was unable to completely inhibit the filamentation of *C. albicans*, in contrast to cocultures under normoxia (4% oxygen ± 0.18 s.d.) (Figures 3a and b). As acyl homoserine lactones (AHLs) are responsible for inhibiting fungal filamentation, we determined the concentrations of these molecules in cocultures and monocultures under normoxic and hypoxic conditions (Supplementary Materials and Methods). Figure 3b shows that the AHL concentration was lower under hypoxic conditions in the presence of *C. albicans*. The low concentrations of AHLs observed in hypoxic cocultures were not caused by a reduced population of bacteria, as the cell density of the cocultures was similar under hypoxia and normoxia ($6.7 \times 10^8 \pm 1.5$ and $7.2 \times 10^8 \pm 1.3$ CFU ml^{-1}). These results suggest that a low oxygen level and the interaction with the fungus are two factors that influence AHL production and therefore alter intraspecific and interspecific communication.

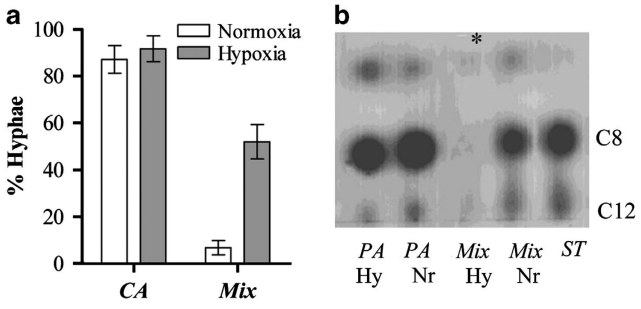

Figure 3 *P. aeruginosa* inhibits the yeast-to-hyphal transition under normoxia but not under hypoxic conditions. (**a**) Percentage of hyphae observed in normoxic and hypoxic cultures after 24 h: CA; *C. albicans*. (**b**) Thin-layer chromatography (TLC) of acyl-HSLs produced by *P. aeruginosa* (PA) in monocultures and cocultures: Hypox, hypoxia and Nor, normoxia. The samples were obtained after 24 h of culture and processed as described in Supplementary Materials and Methods. Acyl-HSL standards: C12, 3-Oxo-C12-HSL, and C8-HSL. The asterisk indicates undetectable acyl-HSLs in a hypoxic coculture. The panels present the means ± s.e.m. ($n = 3$).

It has been shown that low oxygen enhances *C. albicans* filamentation (Dumitru *et al.*, 2004) and that the fungal response to hypoxia overlaps the response to low iron (Synnott *et al.*, 2010), indicating that competition for iron may be greater in hypoxic conditions and may be connected with hyphae formation. A genome-wide transcriptional analysis of morphology determination in *C. albicans* showed that a significant number of genes required for iron utilization were overexpressed during the yeast-hyphal transition (Carlisle and Kadosh, 2013). These data suggest a link between iron metabolism and yeast-hyphal transition; however, this has not been investigated. Taking together these results, we suggest that the low AHLs concentration, hypoxic condition and iron competition may be factors influencing the fungus filamentation in mixed biofilms under hypoxia.

Whole-cell proteome response of C. albicans *and* P. aeruginosa *in coculture*

Proteomics experiments were performed to identify *P. aeruginosa* and *C. albicans* proteins that were differentially regulated in mixed biofilms after 24 h of growth under hypoxic conditions. Bacteria and fungi were grown together in liquid medium under conditions that allowed physical interaction of the two species to form a mixed biofilm on a polystyrene surface (Figure 2c). Fungal and bacterial cells were separated from the mixed biofilms, and protein expression patterns were determined via 2D-PAGE. A total of ~700 spots were detected and analyzed as described in the Materials and methods section (Supplementary Figures S1 and S2). The average protein levels in the spots were quantified, and those showing relative changes in abundance of > 1.5-fold between conditions (increase or decrease) at the 95% confidence level ($P < 0.05$) were considered significantly different.

The interaction with *C. albicans* induced changes in the proteome of *P. aeruginosa*, resulting in differential expression of 163 electrophoretic entities, 108 of which were identified (Supplementary Table S1). In addition, up to 126 electrophoretic entities obtained from *C. albicans* displayed altered expression in response to *P. aeruginosa*, 110 of which were identified (Supplementary Table S2).

The siderophore-mediated iron acquisition system has an essential and dominant role in C. albicans–P. aeruginosa *mixed biofilms*

Siderophores are small, high-affinity iron-chelating compounds secreted by microorganisms in response

to iron limitation. Because the levels of free ferric iron in biological systems are always extremely low (10^{-8} M), siderophores have an important role in microbe–microbe and host-pathogen interactions (Ratledge and Dover, 2000; Harrison *et al*., 2008). For example, *P. aeruginosa* mutants incapable of either siderophore synthesis or siderophore transport are far less virulent than their wild-type counterparts (Takase *et al*, 2000). In the context of microbial interactions, iron competition in *Pseudomonas* has been studied, and the role of the pyoverdine siderophore produced by *Pseudomonas* species has been clearly demonstrated in competition with fungus and bacteria (Loper and Buyer, 1991; Harrison *et al*., 2008). For example, Purschke *et al.* (2012) demonstrated that *P. aeruginosa* increases pyoverdine production in response to iron competition with *C. albicans* in mixed biofilms. Consistent with these findings, we identified proteins with known roles in iron uptake mediated by siderophores that were upregulated in *P. aeruginosa* in mixed biofilms in response to the interaction with *C. albicans*. Among the most highly induced proteins were the group of siderophore receptors constituted by the ferric pyochelin receptor FptA, the hydroxamate-type ferri-siderophore receptor FiuA, the citrate hydroxamate siderophore receptor ChtA, the ferrienterobactin receptor PfeA, the ferripyoverdine receptor FpvA, the alternative type I ferripyoverdine receptor FpvB and the putative TonB-dependent receptor CirA. In addition, the expression of proteins involved in the biosynthesis of the siderophore pyoverdine (PvdH and PvdA) and two proteins (HasR and PhuR) required for heme and hemoglobin uptake was highly induced in mixed biofilms (Supplementary Table S1).

Supporting the above results, we observed that the pyoverdine production per cell was significantly

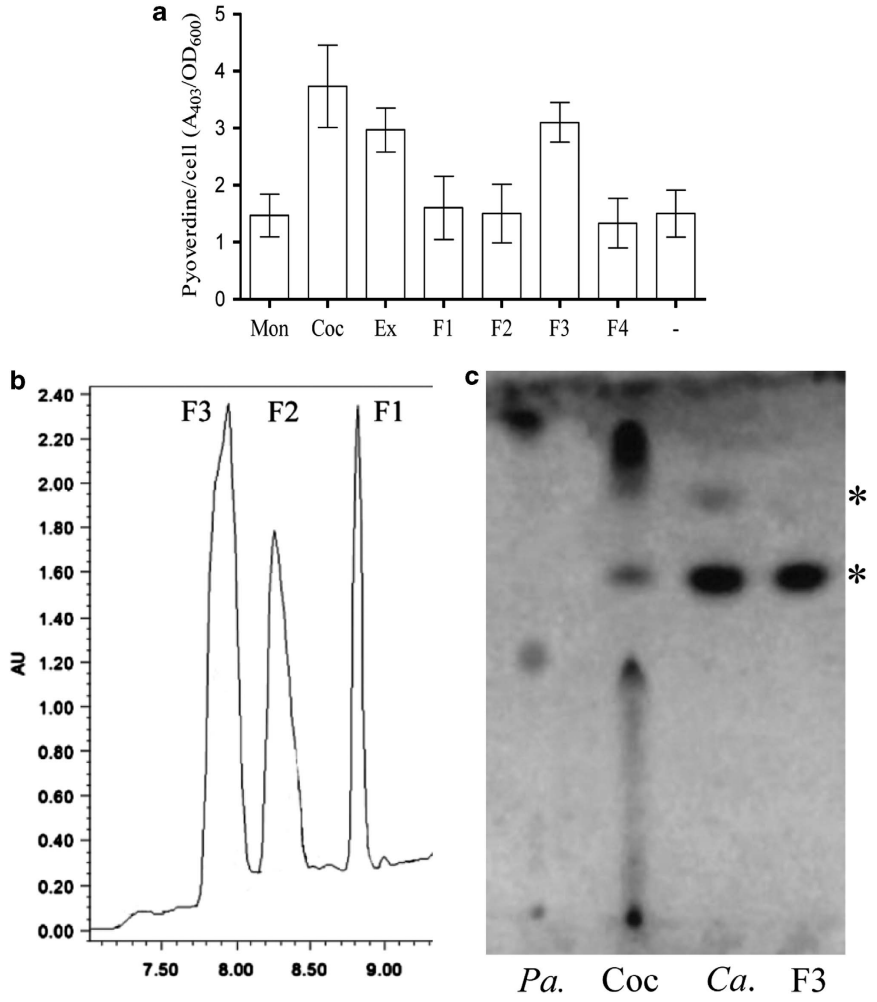

Figure 4 The production of pyoverdine is increased in mixed biofilm. (**a**) Pyoverdine production under *P. aeruginosa* monoculture (Mon), coculture (Coc), *P. aeruginosa* monoculture + a methanolic extract from *C. albicans* supernatant (Ex) and *P. aeruginosa* monoculture + HPLC (high-performance liquid chromatographic) fractions of ethyl acetate extracts from *C. albicans* supernatants (F1-F4) and solvent control—ethyl acetate (−). All experiments were conducted in hypoxia. (**b**) Chromatogram of an ethyl acetate extract from the *C. albicans* supernatant. (**c**) TLC to detect siderophores. Ethyl acetate extracts were obtained from *P. aeruginosa* monocultures (*Pa.*), cocultures (Coc) and *C. albicans* monocultures (*Ca.*). *C. albicans* phenolates are indicated by asterisks. The panels present the means ± s.e.m. ($n = 3$).

increased in the presence of *C. albicans* (Figure 4a). We tested the ability of *C. albicans*-conditioned medium to induce pyoverdine production. We observed that a methanol extract of the culture supernatant increased the production of pyoverdine (Figure 4a). The extracts were fractionated through high-performance liquid chromatography (Figure 4b). A total of 12 fractions were evaluated, one of which, fraction 3 (F3), showed the capacity to induce the synthesis of pyoverdine (Figure 4a). Further experiments will be needed to determine the molecular structure of the active compound in this fraction. However, the preliminary results suggest that it contains phenolate molecules (Figure 4c). We propose that these molecules are produced by *C. albicans* to capture iron and may function as siderophores.

We hypothesized that iron competition has an important role in the development of the interaction. To examine this hypothesis, we evaluated the effect of iron supplementation on the growth of the fungus and bacterium. We observed that iron supplementation significantly increased the growth of the bacterium in both mixed and pure cultures (Figure 5a). In contrast, in *C. albicans* monocultures, iron supplementation did not increase the growth of the fungus (Figure 5b), suggesting that the iron content in the unsupplemented culture is sufficient to support maximal growth of the fungus but not that of the bacterium. In the iron-supplemented medium, an early antagonistic interaction was established, in which the rapid growth of the bacterium eliminated the fungal population (Figure 5b).

As yeast mutants deficient in iron utilization were unable to compete with *P. aeruginosa* in mixed biofilms (Supplementary Figure S3), we propose that the iron uptake ability of the fungus is a determining factor for the establishment of the interaction. Thus, these results indicate that, in mixed biofilms, the competence for iron uptake determines the nature and dynamics of the interaction.

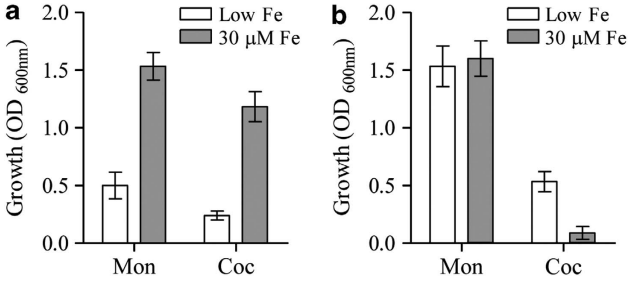

Figure 5 Effects of iron on *P. aeruginosa* (**a**) and *C. albicans* (**b**) growth. Monocultures (Mon) and cocultures (Coc). Growth was determined after 24 h of culture. To quantify the growth of *C. albicans* and *P. aeruginosa* in cocultures, cells (including planktonic and sessile cells) were separated on a Percoll gradient (Supplementary Materials and Methods) and were suspended in 20 ml water. Optical density (OD600) for each species was determined. The panels present the means ± s.e.m. ($n = 3$).

Iron-regulated virulence factors of P. aeruginosa *are overexpressed in mixed biofilms*

Lamont *et al.* (2002) demonstrated that in addition to its role as an iron scavenger, the siderophore pyoverdine acts as a signaling molecule to control the production of at least three virulence factors (exotoxin A, an endoprotease and pyoverdine itself) that are major contributors to the ability of *P. aeruginosa* to cause disease. As pyoverdine production was increased in the mixed biofilms (Figure 4a), we hypothesized that the bacterium shows increased virulence when competing for iron with the fungus. To test this hypothesis, we analyzed the extracellular proteins present in the cocultures and monocultures.

In *C. albicans* monocultures, the concentration of total extracellular protein was significantly lower than the concentration detected in monocultures of *P. aeruginosa* (Supplementary Figure S4). In our analysis of coculture supernatants, we were not able to identify *C. albicans* proteins, which could be due to slow production of extracellular proteins by *C. albicans* and bacterial protease activity. Extracts of secreted proteins were analyzed via 2D-PAGE, and a total of ~300 spots were detected, 75 of which were differentially expressed, and 55 of these were identified (Supplementary Table S3 and Supplementary Figure S4).

As previously reported by Purschke *et al.* (2012), we observed a significant increase in the production of iron-regulated proteases by *P. aeruginosa* in coculture supernatants, including PrpL, PasP, AprA and a hypothetical protein zinc-protease, PA0572 (Supplementary Table S3) (Wilderman *et al.*, 2001; Marquart *et al.*, 2005). These proteins are known for their strong proteolytic activities, which are responsible for rupturing tight junctions in the epithelium, leading to tissue invasion and bacterial spreading (Azghani *et al.*, 1993). In contrast, the quorum-sensing-regulated proteases, including LasB and PepB, and a chitin-binding protein were downregulated in the cocultures, which is consistent with the low concentration of AHLs detected in cocultures under hypoxic conditions (Figure 3b).

Interestingly, we observed a significant increase in the production of PvdQ in the coculture supernatant (Supplementary Table S3). PvdQ is an iron-regulated AHL acylase that degrades long-acyl but not short-acyl AHLs (Sio *et al.*, 2006; Nadal *et al.*, 2010). This result may provide an explanation for the previous finding that the level of long-acyl AHLs is greatly reduced in cocultures as compared with *P. aeruginosa* pure cultures (Figure 3b).

Kirienko *et al.* (2013) demonstrated that *P. aeruginosa* does not require quorum-sensing pathways to kill *Caenorhabditis elegans*. Supporting a role for iron in *P. aeruginosa* pathogenesis, pyoverdine was found to be required to cause hypoxia and death in *C. elegans*.

The increased virulence was confirmed by our observation of an enhancement of the proteolytic activity of coculture supernatants against human serum proteins and against the adhesion of HeLa cells (Figures 6a and b). We propose that a significant increase of iron-regulated proteases could be a bacterial strategy for affecting the viability of *C. albicans* and causing release of iron from fungal proteins.

Consistent with a role for pyoverdine as a signaling molecule that controls the production of virulence factors, we observed an increased concentration of Exotoxin A in coculture supernatants (Supplementary Table S3), which is one of the most powerful extracellular virulence factors produced by *P. aeruginosa* (Iglewski and Kabat, 1975).

To obtain iron *in vivo*, bacteria additionally or alternatively use other mechanisms. They obtain iron directly from host iron sources via specific outer membrane receptors that bind free heme (Ochsner *et al*., 2000). In mixed biofilms, we detected two secreted proteins from the heme-acquisition pathway, HasA and HasR. These proteins are considered to be important virulence factors that have a role in colonization, survival, tissue invasion and in the ultimate damage caused to the host.

Taking these results together, we suggest that competition for iron is the main mechanism that triggers the virulence of *P. aeruginosa* in mixed biofilms. In this manner, iron competition has the potential to cause significant 'collateral damage' to the host and determines the course and severity of a mixed infection.

The production of small molecules regulated by the quinolone signaling system is increased in mixed biofilms

Rhamnolipids and pyocyanin (a phenazine pigment) are important *P. aeruginosa* virulence factors. These compounds are known for their toxicity in other bacteria (Baron and Rowe, 1981) and in eukaryotes ranging from nematodes to humans (Ran *et al*., 2003; Lau *et al*., 2004; Mavrodi *et al*., 2006; Morales *et al*., 2010; Morales *et al*., 2013).

We observed a significant increase in the concentration of rhamnolipids and pyocyanin in the coculture supernatants (Figures 6c and d). The production of rhamnolipids in *P. aeruginosa* is controlled by both the LasR/3-oxo-C12-HSL quorum-sensing signaling pathway and the MvfR/PQS/PqsE quinolone signaling system, which uses 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS) (Diggle *et al*., 2003). Is known that iron limitation promote the production of rhamnolipids, because the expression of genes for rhamnolipids biosynthesis and their transcriptional regulator *rhlR*, are enhanced by iron limitation (Bredenbruch *et al*., 2006).

Similar to AHLs, PQS regulates the production of virulence determinants, including rhamnolipids, pyocyanin, elastase and the galactophilic lectin LecA, in addition to influencing biofilm development (Pesci *et al*., 1999; Diggle *et al*., 2003).

As we observed that there was a reduced concentration of AHLs in the cocultures (probably due to upregulation of PvdQ triggered by iron competition), we propose that, in mixed biofilms, the increased production of rhamnolipids and pyocyanin is due to the activation of the PQS

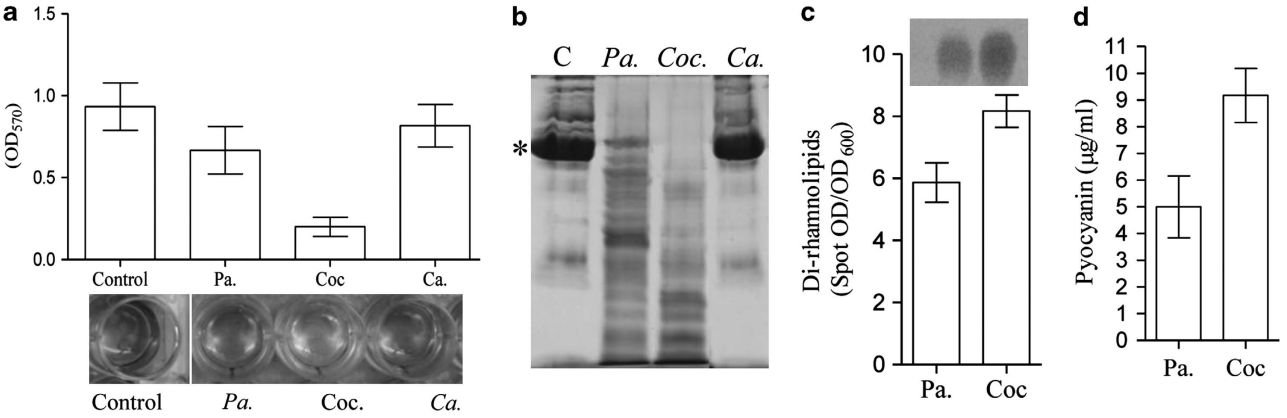

Figure 6 Assays of cytotoxic activity of microbial culture supernatants and production of *P. aeruginosa* virulence factors. (**a**) Effect of supernatants on HeLa cells adherence. Cells were incubated with *P. aeruginosa* monoculture supernatants (*Pa.*), coculture supernatants (Coc.), *C. albicans* monoculture supernatants (*Ca.*) or phosphate-buffered saline (PBS) buffer (Control). The adhered cells were measured by crystal violet staining (DO_{570}). (**b**) Degradation of serum proteins after exposure to microbial culture supernatants. Serum was incubated with *P. aeruginosa* monoculture supernatants, coculture supernatants, *C. albicans* monoculture supernatants or PBS buffer. The reactions were analyzed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The figure shows a representative image from three independent experiments. Albumin (asterisk) was totally degraded in the coculture supernatant. In contrast, the *C. albicans* supernatant showed no effect on albumin. (**c**, **d**) The production of rhamnolipids and pyocyanin is increased in mixed biofilms. Panel (**c**) shows rhamnolipid relative concentration in supernatants. Thin-layer chromatographic (TLC) analysis of *P. aeruginosa* rhamnolipid biosynthesis in *P. aeruginosa* monocultures (Pa.) and cocultures (Coc). Di-rhamnolipid production was measured by dividing the optical density (OD) of the spots in the TLC with the *P. aeruginosa* cell density (OD_{600}). The bacteria cell density was determined as described in the legend of Figure 4. (**d**) Pyocyanin concentrations in culture supernatants.

signaling pathway rather than the LasR/3-oxo-C12-HSL pathways. PQS has been demonstrated to induce the expression of genes associated with the iron-deprivation pathway, in which the iron-chelating activity of PQS induces pyoverdine production (Bredenbruch *et al.*, 2006; Diggle *et al.*, 2007).

Consistent with the observed increase in the production of pyocyanin and rhamnolipids, we observed an increase in the concentration of PQS in the coculture supernatants (Supplementary Figure S5), which supports our hypothesis that the PQS signaling pathway is active in mixed biofilms. The finding that *C. albicans* increases production of PQS in mixed biofilms, even in the absence of increased AHLs, is consistent with previous reports (Cugini *et al.*, 2010). We propose that the accumulation of PQS in the cocultures occurs because iron competition induces the expression of PrrF (a small regulatory RNA expressed under low-iron conditions), which represses gene-encoding enzymes involved in the degradation of anthranilate, a precursor of PQS (Oglesby *et al.*, 2008). Another proposal is that in mixed biofilms *C. albicans*-produced farnesol stimulates PQS production in *P. aeruginosa* (Cugini *et al.*, 2010).

Drug-resistance proteins and other outer membrane proteins are overexpressed in mixed biofilms

In mixed biofilms, *P. aeruginosa* overexpress the porin OprC, OprE, OprF, OprQ and outer membrane proteins, such as OprL, Opr86 and OpmH (Supplementary Table S1). These outer membrane proteins are key molecules that participate in the interaction between the cell and its environment. In addition, it has been demonstrated that these proteins are intrinsic participants in antibiotic resistance (Hancock and Brinkman, 2002). OprC, OprL and GroEL are necessary for biofilm formation, drug resistance and for adaptation to anaerobic conditions (Yoneyama *et al.*, 1995). It is possible that the observed overexpression of outer membrane proteins involved in drug resistance and stress is a response to environmental changes generated by *C. albicans*.

P. aeruginosa *shows increased mutability during the interaction with* C. albicans

P. aeruginosa possesses three catalases, KatA, KatB and KatC. KatA is the catalase that shows primary activity in all growth phases. KatB and KatC are induced when cells are exposed to hydrogen peroxide and other components that generate superoxide radicals (Brown *et al.*, 1995). Ma *et al.* (1999) demonstrated that, under decreased concentrations of iron, the expression and activity of KatA are strongly reduced. We observed that, in mixed biofilms, KatA was downregulated, which could be a consequence of the iron-deficient conditions. Furthermore, we observed that an alkyl hydroperoxidase (AhpC) and a putative peroxidase (PA3529) were downregulated in mixed biofilms. To validate these results, we measured the total catalase activity of *P. aeruginosa* in mixed and monospecific biofilms. We observed that the specific activity of these enzymes was 49.66% ± 15.69 s.d. lower in mixed biofilms with respect to monospecific biofilms (Supplementary Figure S6). Driffield *et al.* (2008) observed that the expression of KatA, AhpC and PA3529 were reduced in biofilms, and the frequency of ciprofloxacin-resistant mutants was increased in sessile cells with respect to planktonic cells. These authors suggested that the downregulation of antioxidant enzymes in *P. aeruginosa* biofilms may enhance the rate of mutagenic events owing to accumulation of DNA damage.

To know the effects of interspecific interaction in *P. aeruginosa* mutability, we used the rifampicin-resistance method (Oliver *et al.*, 2000; Weigand and Sundin, 2012). Mutations responsible for Rif^R map exclusively to rpoB and produce amino-acid substitutions in three primary regions of the β subunit of RNA polymerase (Garibyan *et al.*, 2003; Jatsenko *et al.*, 2010). The 38 diverse mutational possibilities in *rpoB* available to achieve resistance include all transition and transversion substitutions, providing a suitable system for analyzing mutability. We observed a significant increase in the frequency of Rif^R mutants in cocultures with respect to monocultures in two different culture media (Figure 7a). To confirm that the Rif^R mutants have an independent genotype and arise by an increase in mutation rate, we analyzed phenotypic traits and genotype (sequencing *rpoB*) of 30 Rif^R mutant isolates of 24-h-old cocultures. Our results show that 27 mutants differ in at least one trait (Supplementary Table S4).

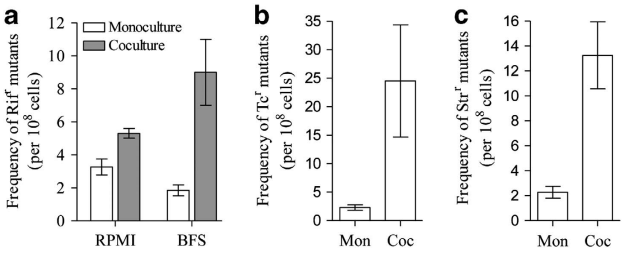

Figure 7 Frequency of spontaneous mutants resistant to antibiotics in *P. aeruginosa* cells collected from monoculture (Mon) and coculture (Coc) growth in hypoxia. (**a**) Frequency of mutants resistant to rifampicin (Rif^R), (**b**) tetracycline (Tc^R) and (**c**) streptomycin (Str^R). Frequency of Rif^R mutants was estimated to bacterial population growth in RPMI medium and fetal bovine serum. Frequency of Tc^R and Str^R mutants was estimated to bacterial population growth in RPMI. Dilutions of 24 h old cultures were spread on LB plates containing 100 μg ml^{-1} Rif, 20 μg ml^{-1} tetracycline or 20 μg ml^{-1} streptomycin. The plates were incubated at 37 °C, and resistant colonies were scored 48 h after plating. The dilutions were also plated on LB plates without antibiotics to determine the total number of CFUs. The mutation frequencies were approximated as the mean number of antibiotic-resistant cells divided by the total number of CFUs. Results shown represent the average of three independent experiments ± s.e.m.

In addition, by competition experiments between wild and mutant strain, we found that the Rif^R mutants do not have a growth advantage in cocultures (Supplementary Figure S7). These results demonstrate that the increased incidence of Rif^R mutants arise by an increase in mutation rate and not by having a growth advantage in cocultures. Consistent with these results, we observed a significant increase in the frequency of *P. aeruginosa* mutants resistant to streptomycin and tetracycline in cocultures (Figures 7b and c). We propose that iron deficiency and the increased production of oxidants (for example, pyocyanin) cause a decrease in total catalase activity in mixed biofilms and thereby increase oxidative stress, which, in turn, increase mutation events, enhancing the mutability of *P. aeruginosa*. Therefore, interespecific competition can promote the rapid emergence of antibiotic-resistant mutants among bacterial populations during infections.

Ecological mechanisms maintaining biodiversity seem to be diverse themselves. We suggest that competition between species is an ecological process that can influence the evolution (Czaran *et al*., 2002) of microbial virulence, and consequently, it is expected that the evolutionary response (rapid evolution) alters the dynamics of microbial-microbial–host interactions (Andrade-Domínguez *et al*., 2013).

Differential expression of C. albicans *iron-regulated proteins indicate iron competence in mixed biofilms*

Consistent with our hypothesis that in mixed biofilms fungus and bacteria compete for iron, we observed in mixed biofilms, relative to the monospecific biofilms, downregulation of *C. albicans* iron-regulated proteins. These included proteins involved in a variety of iron-dependent processes, such as aerobic respiration (Aco1p, Idh2p), the respiratory electron transport chain (Qcr2p) heme biosynthesis (Hem13p and Hem15p) and haem-containing proteins (Cat1p). A similar iron-regulated pattern of *ACO1*, *IDH2*, *HEM13* and *CAT1* gene expression has been observed in *C. albicans* when iron is limited (Lan *et al*., 2004; Chen *et al*., 2011).

Interestingly, we found that aconitase and catalase were also downregulated in *P. aeruginosa* during interaction with the fungus, showing a conserved pattern in the regulation of these proteins in response to iron competition.

C. albicans Hap43p is a repressor that is induced under low-iron conditions and is essential for iron-responsive transcriptional regulation and virulence (Hsu *et al*., 2011). Consistent with downregulation of *C. albicans* iron-regulated proteins in mixed biofilms, we found that 19.71% (16) of differentially expressed proteins in mixed biofilms are Hap43p regulated (Supplementary Table S2). This suggests important roles for Hap43p transcription factor in the regulation of iron homeostasis in *C. albicans* during the interaction with *P. aeruginosa*.

C. albicans *proteins associated with virulence and drug resistance are upregulated in mixed biofilms*

In *C. albicans*, many secreted hydrolytic enzymes are potential virulence factors that are located at the cell surface before their secretion and are thought to be involved in the adhesion to/invasion of host tissues (Naglik *et al*., 2003). We detected a neutral arginine, alanine, leucine-specific metallo-aminopeptidase (Ape2p) that was upregulated in mixed biofilms. Klinke *et al.* (2008) suggest that this aminopeptidase has the potential to contribute to the pathogenicity of *C. albicans*. In addition, a 1,3-beta-glucosyltransferase (Bgl2p), which has a virulence role in mouse systemic infection (Sarthy *et al*., 1997), was also overexpressed (Supplementary Table S3). Upregulation of these proteins in mixed biofilms may have a synergistic interaction with the virulence factors of the bacterium, resulting in enhanced pathogenesis.

Alterations in glucose metabolism in C. albicans *during the interaction with* P. aeruginosa

Glycolytic enzymes are transcriptionally regulated in response to environmental conditions, such as oxygen levels and carbon sources and availability, and in response to cellular demands, such as metabolite concentrations and energy needs (Chambers *et al*., 1995). We observed that, in mixed biofilms, *C. albicans* overexpressed hexokinase isoenzyme 2, Hxk2p, which has been demonstrated to be induced in the presence of glucose (Askew *et al*., 2009). In contrast, other proteins involved in glycolysis/gluconeogenesis (Tdh3p and Pgk1p) and pyruvate metabolism (Pdc11p, Pda1p and Lpd1p) were downregulated, suggesting that glucose metabolism was altered by the interaction with *P. aeruginosa* (Figure 8). Consistent with these results, the glucose concentration at 24 h in cocultures was 98% ($\pm$ 1.2 s.d.) of the total supplied, whereas that detected in *C. albicans* monocultures was 1% ($\pm$ 0.07 s.d.) of the total supplied. Although glucose consumption was limited in the cocultures, fungal growth was still observed (Figure 5b), which suggests that *C. albicans* used other carbon sources for growth. In contrast, amino acids such as glutamine, aspartate and arginine were depleted in the cocultures (Supplementary Table S5). We found that supplementation with glutamine significantly increased the growth of *C. albicans* in mixed biofilms, but not in monospecific biofilms (Supplementary Figure S8), indicating that *C. albicans* used amino acids as an alternative carbon source in mixed biofilms. These results indicate that the glucose metabolism of *C. albicans* was affected by the interaction with *P. aeruginosa* (Figure 8). Because we observed that, in mixed biofilms, *C. albicans* was exposed to pyocyanin, we hypothesized that this metabolite secreted by the bacterium was responsible for the inhibition of glucose consumption by the fungus. To verify this hypothesis, we exposed

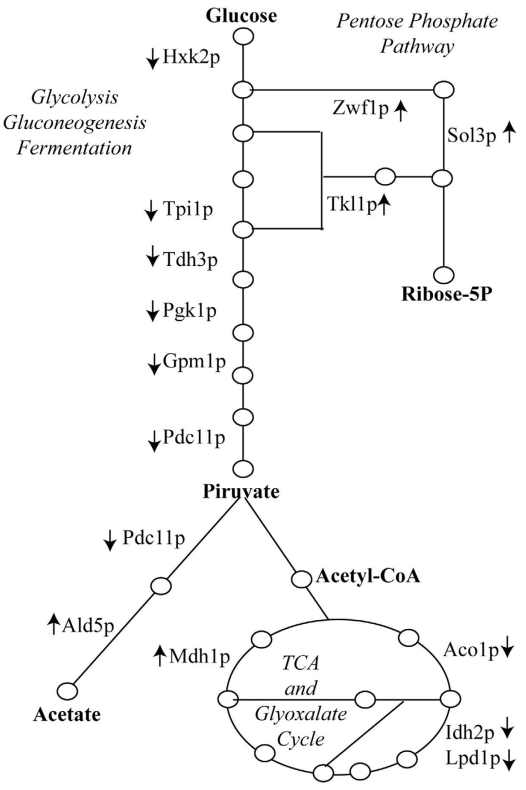

Figure 8 There was a widespread repression of glycolysis, pyruvate metabolism and the TCA cycle of *C. albicans* in mixed biofilms. In contrast, proteins involved in the pentose phosphate pathway were upregulated. A schematic representation of the pathways is presented.

C. albicans cells to concentrations of commercial pyocyanin similar to those detected in the mixed biofilms ($10\,\mu g\,ml^{-1}$) and assayed the resultant glucose consumption. We observed that pyocyanin inhibited 90% (± 5 s.d.) of glucose consumption and affected the growth of *C. albicans* (Supplementary Figure S9). Price-whelan *et al.*, (2007) demonstrated that pyocyanin alters the redox balance and the flow of carbon through the central metabolic routes in *P. aeruginosa*. These authors proposed that the superoxide radicals generated by pyocyanin can reduce the sulfhydro groups of the lipoamine cofactor from the E2 subunit of pyruvate dehydrogenase, resulting in inhibition of enzyme activity.

C. albicans *respiration proteins are downregulated in mixed biofilms*

In mixed biofilms, we observed downregulation of important proteins required for the tricarboxylic acid cycle in *C. albicans* (the aconitase Aco1p, isocitrate dehydrogenase Idh2 and malate dehydrogenase Mdh1p). A previous study on *C. albicans* demonstrated that these enzymes are repressed when cells are cultured under low-iron conditions (Lan *et al.*, 2004). Aconitate dehydrogenase contains an Fe–S (iron–sulfur) group and shows activity that is dependent on the concentration of this metal; therefore, there is a reduction of the flow of carbon from the Krebs cycle under increased iron conditions (Shakoury-Elizeh *et al.*, 2010). We also detected downregulation of the ubiquinol–cytochrome *c*–reductase Qcr2p. This protein is essential in the mitochondrial respiratory chain and is repressed under iron-deficient conditions and when there is a lack of heme groups (Dorsman and Grivell, 1990). In addition to the repression of these enzymes, it is possible that the Krebs cycles of *C. albicans* in mixed biofilms is affected by pyocyanin (Figure 8), which has been demonstrated to block respiration by inhibiting the activity of aconitase and affect the membrane potential of mitochondria from human cells (O'Malley *et al.*, 2003).

Formate metabolism

We observed that the formate dehydrogenase Fdh1p was overexpressed in mixed biofilms but undetectable in *C. albicans* monocultures. It has been proposed that Fdh1p participates in the generation of energy through the glyoxylate cycle and beta oxidation of lipids (Prigneau *et al.*, 2003 and Kusch *et al.*, 2008). In plants and in bacteria, the formate pathway is induced by iron deficiency or anaerobiosis, and it has been proposed that this pathway provides a source of NADPH for the cell (Francs-Small *et al.*, 1993). We propose that (A) the overexpression of Fdh1p observed in mixed biofilms suggests that *C. albicans* is obtaining energy through alternative metabolic routes, because it is unable to utilize the available glucose in the medium, and (B) the low levels of iron detected in mixed biofilms are potentially factors that induce the overexpression of Fdh1p in *C. albicans*.

Response of C. albicans *to oxidative stress and to alterations in the redox equilibrium*

The pentose phosphate pathway is of great importance for the generation of NADPH and substrates for the biosynthesis of nucleic acids and amino acids (Miosga and Zimmermann, 1996). It is also important in the protection of cells against oxidative stress, as NADPH is a cofactor of antioxidant enzymes (Minard and McAlister-Henn, 2001). We identified two proteins from the non-oxidative branch of the pentose phosphate pathway overexpressed in mixed biofilms: the transaldolase Tal1p and the transketolase Tkl1p (Supplementary Table S2 and Figure 8). Both enzymes are overexpressed under conditions of oxidative stress in *C. albicans* (Wang *et al.*, 2006). As phenazines, such as pyocyanin, inhibit normal fungal respiratory activity (Morales *et al.*, 2013), it is likely that activation of the pentose phosphate pathway contributes to maintaining the metabolic activity and redox equilibrium of *C. albicans* (Figure 8).

Other proteins related to oxidative stress were found to be overexpressed by *C. albicans* in mixed

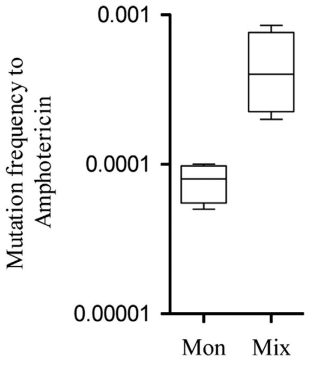

Figure 9 *C. albicans* mutation frequency following exposure to 100 μg ml^{-1} Amphotericin. Cells were collected from monospecific biofilms (Mon) and mixed biofilms (Mix). Mutation frequencies were expressed as the number of Amphotericin-resistant mutants recovered as a fraction of the viable count (Supplementary Materials and Methods). The panel presents the means ± s.e.m. ($n = 4$).

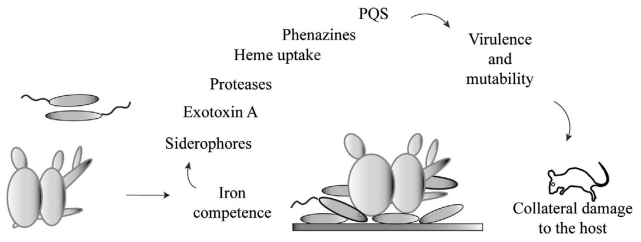

Figure 10 An abstract model depicting how interspecies competition triggers *P. aeruginosa* virulence and mutability in mixed biofilms. We propose that in mixed biofilms, the fungus and bacterium compete for iron, which triggers the expression of iron-regulated virulence factors in *P. aeruginosa*, for example, the siderophores pyoverdine and pyochelin, exotoxin A, proteases and heme-binding proteins. In addition, in mixed biofilms, *P. aeruginosa* shows increased production of the cytotoxic molecules pyocyanin, rhamnolipids and PQS. The decrease of the activity of antioxidant proteins caused by iron deficiency in both species may be the cause of the increase in the frequency of mutant cells in mixed biofilms.

biofilms, including a chaperone from the HSP70p family, the heat-shock protein Hsp78p, alkyl hydroperoxide peroxidase Tsa1p and the putative alkyl hydroperoxidase Ahp1. In contrast, the catalase Cat1p and chaperones Kar2p, Ssa2p, Ssb1p and Ssz1p were downregulated in mixed biofilms (Supplementary Table S2). This downregulation could be caused by the reduced iron levels, as it has been demonstrated that these proteins are downregulated in low-iron conditions (Lan *et al.*, 2004). Based on the observed reduction of the activity of the most important enzyme for detoxifying oxygen radicals and the oxidative damage generated by pyocyanin, it is expected that in mixed biofilms, the fungus undergoes oxidative stress, which may increase mutation events and thus the emergence of hypermutator cells. This hypothesis is supported by our observation of a significant increase in the frequency of amphotericin-resistant cells in mixed biofilms (Figure 9).

The P. aeruginosa–C. albicans *interaction resulted in a strong synergistic effect on mouse mortality*

Mixed-species infections can have consequences that differ from those associated with single-species infections, increasing the virulence of the organisms involved in the co-infection. To verify this hypothesis for interaction studied here, immunodeficient Nu/Nu nude mice were co-infected intraperitoneally with sublethal inocula of *P. aeruginosa* PAO1 ($\sim 2 \times 10^7$ cells ml^{-1}) and *C. albicans* CAI-4 ($\sim 5 \times 10^6$ cells ml^{-1}) as described in Supplementary Materials and Methods. The co-infected mice showed a high mortality rate after 12 h with respect to the groups inoculated with *P. aeruginosa* (2×10^7 cells ml^{-1}) or *C. albicans* ($\sim 5 \times 10^6$ cells ml^{-1}) (Supplementary Figure S9). Doubling the dose of *C. albicans* (10^{7}cells ml^{-1}) did not result in mortality of the mice after 2 weeks of observation. In contrast, doubling the dose of *P. aeruginosa* (4×10^7cells ml^{-1}) caused 100% mortality within 12 h (Supplementary Figure S10). These results suggest that *P. aeruginosa* was the cause of death in the co-infected mice.

Roux *et al.* (2009) demonstrated that *C. albicans* impairs macrophage function and facilitates *P. aeruginosa* pneumonia in rats. Overall, these results suggest that *C. albicans* promotes the pathogenicity of *P. aeruginosa* in mixed infections. Previously, Roux *et al.* (2009) reported that the pseudomonal proteolytic enzyme elastase (LasB; also known as pseudolysin) is responsible for the increased virulence of *C. albicans*, but the details of this mechanism remain unclear. In a similar study using a rat pneumonia model, the same group observed that rats administered a subclinical dose of *P. aeruginosa* developed pneumonia only in the presence of viable *C. albicans* (Roux *et al.*, 2009).

Microbes competing with unrelated or distantly related species for limited resources in the same niche activate mechanisms, such as secretion of proteins or small molecules, to attack the competing strains or species (Czaran *et al.*, 2002; Be'er *et al.*, 2009). Because we found that in mixed biofilms *C. albicans* was attacked by *P. aeruginosa* with mechanisms similar to those used to cause harm to their mammalian hosts, we propose that the synergism observed in coinoculated mice was caused by the increased virulence of the bacteria as a result of competition with the fungus for iron (Figure 10).

Conclusions

Microbes involved in polymicrobial infections often display synergistic interactions that result in enhanced pathogenesis. However, the molecular mechanisms governing these interactions are not well understood. Our results provide compelling mechanistic evidence that interspecies competition

between opportunistic pathogens can alter the course of pathogenesis in polymicrobial communities and enhance the selection of mutator cells.

Conflict of Interest

The authors declare no conflict of interest.

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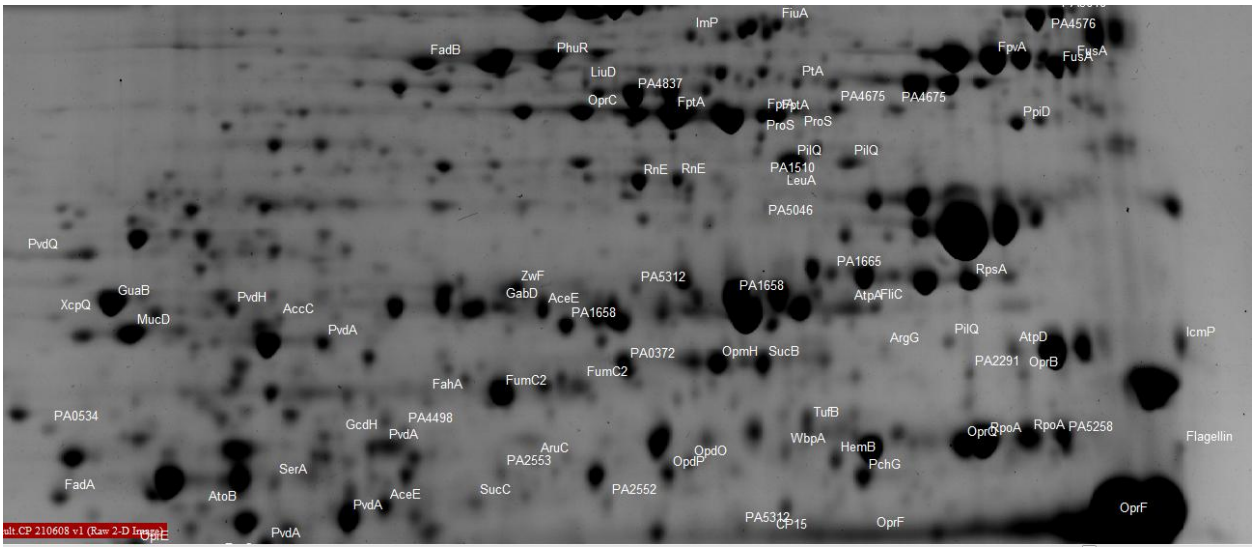
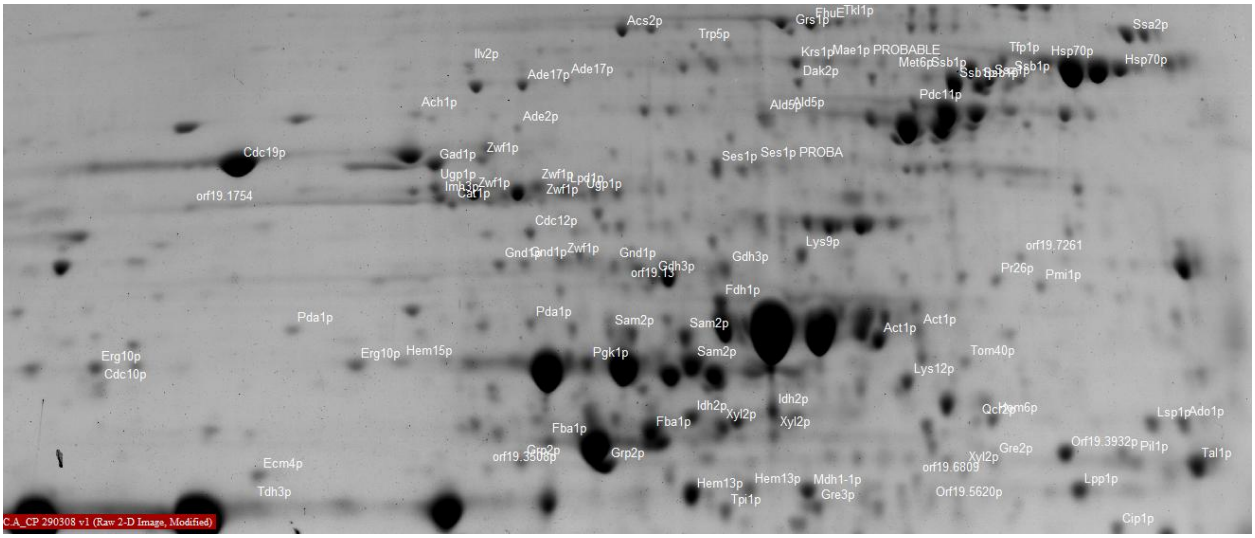
Supplementary Information accompanies this paper on The ISME Journal website (http://www.nature.com/ismej)

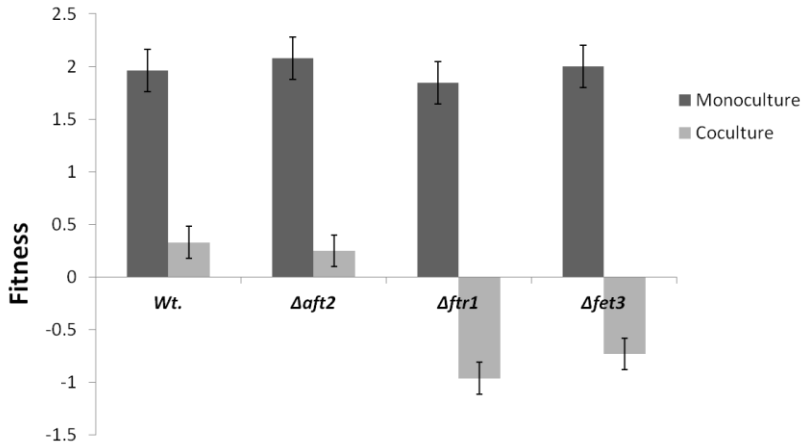
Supplementary figures S1-S10.



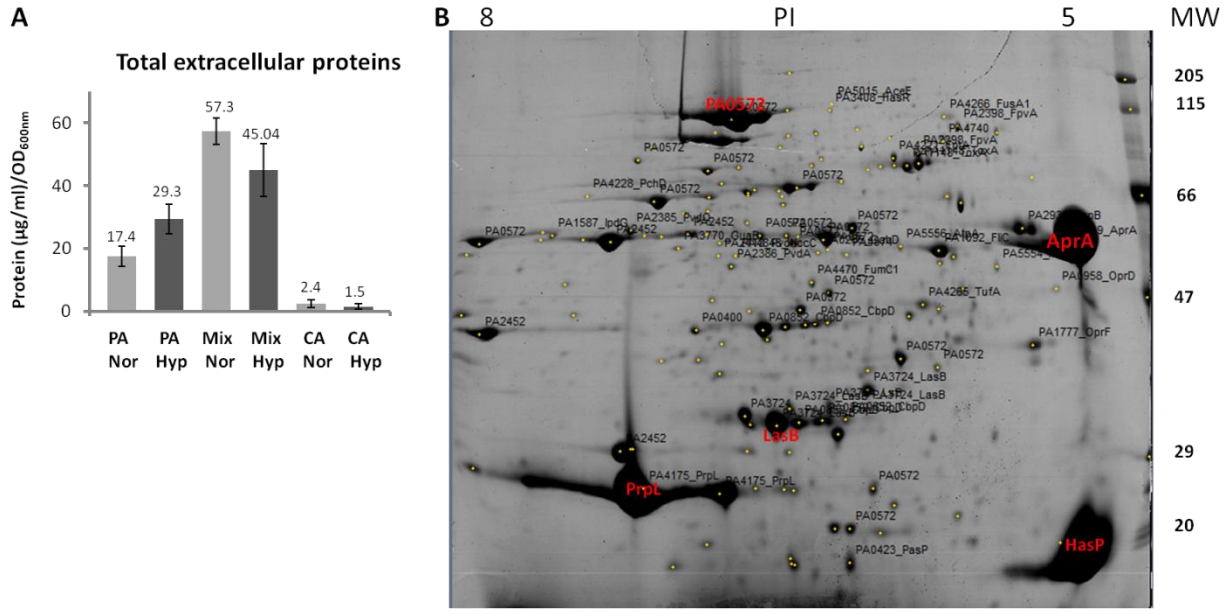
Supplementary Figure S1. Segment of a two-dimensional electrophoresis Coomassie blue-stained gel showing differentially expressed *P. aeruginosa* proteins following interaction with *C. albicans*, identified through mass spectrometry (MALDI-TOF). The proteins were annotated in the gel based on protein names according to the Pseudomonas Genome Database v2.



Supplementary Figure S2. Segment of a two-dimensional electrophoresis Coomassie blue-stained gel showing differentially expressed *C. albicans* proteins following interaction with *P. aeruginosa*, identified through mass spectrometry (MALDI-TOF). The proteins were annotated in the gel based on protein names according to the Candida Genome Database.

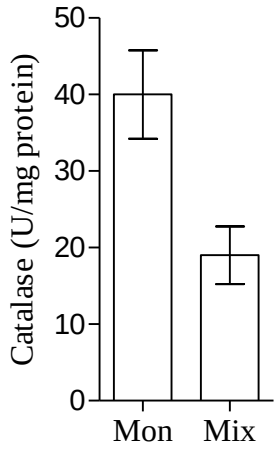


Supplementary Figure S3. *S. cerevisiae* carrying mutations in high-affinity iron permease (*Δftr1*) and cell surface ferroxidase (*Δfet3*) were unable to compete with *P. aeruginosa* in cocultures. Absolute yeast fitness (m) was measured in monocultures and cocultures. The panels present the means ± S. E. M. (n= 3).

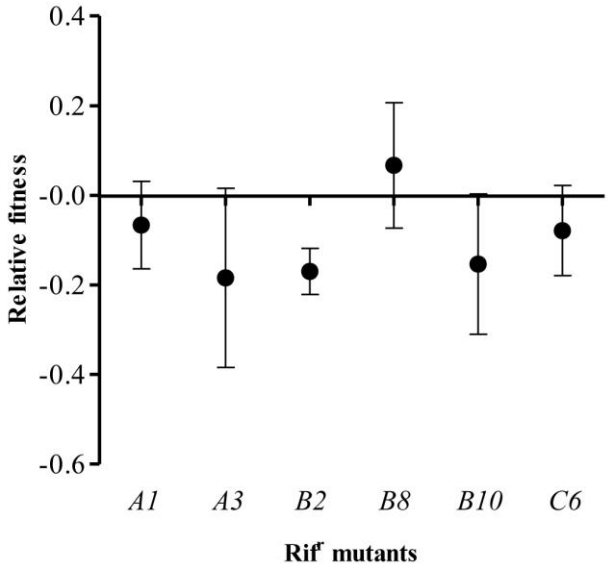


Supplementary Figure S4. A) Total *extracellular protein production* in cocultures and monocultures of *P. aeruginosa* and *C. albicans* after 24 hours. **B**) Two-dimensional gel electrophoresis of extracellular *P. aeruginosa* proteins from mixed biofilms. The HasP, AprA and PrpL proteins represent the major components of the supernatants obtained under different culture conditions. The proteins were annotated in the gel based on protein names according to the Pseudomonas Genome Database v2.

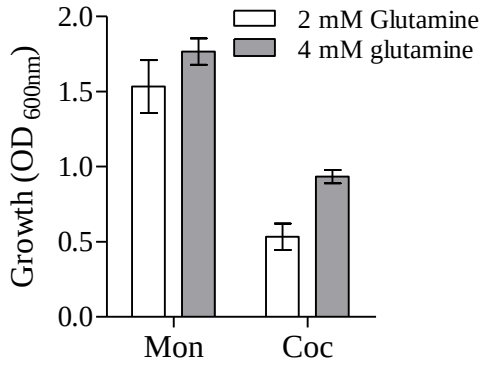
Supplementary Figure S5. TLC assay for PQS (arrow). TLC plate run with supernatant extracts from *P. aeruginosa* (*Pa*.), cocultures (Coc.) and *C. albicans* (*Ca*.). TLC was run using a mixture of dichloromethane:methanol (95:5) as the mobile phase and visualized using a UV transilluminator at 312 nm, and photographs were obtained at this point.



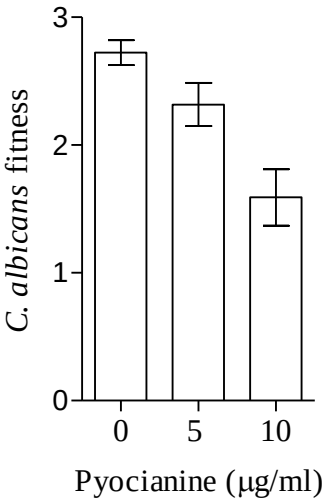
Supplementary Figure S6. Total catalase activity in *P. aeruginosa* cells isolated from monospecific biofilms (Mon) and mixed biofilms (Mix).



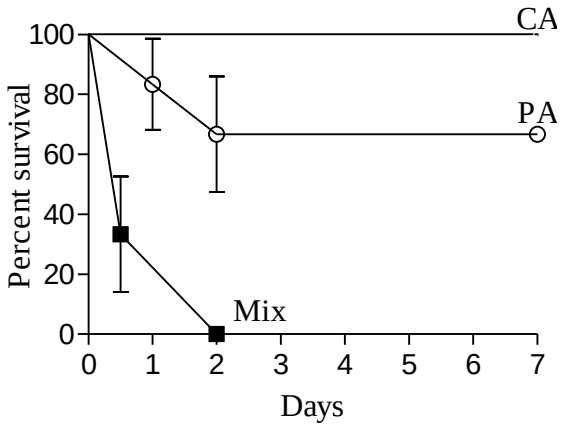
Supplementary Figure S7. the Rif^R mutants have not a growth advantage in cocultures. The relative fitness of the Rif^R genotype was determined relative to *P. aeruginosa* wild-type (initial ratio 1:1) in monocultures and coculture with *C. albicans*. Fitness is given as the difference in Malthusian parameters (*m*). A fitness of zero indicates that the two genotypes are equally fit. The competitive fitness was determined during a single 24-hour growth period. Wild-type strain and the competing Rif^R mutant were differentiated by rifampicin resistance. The panel show the means ±S. E. M. (n= 3).



Supplementary Figure S8. Positive effects of glutamine on *C. albicans* growth. Monocultures (Mon) and cocultures (Coc). Growth was evaluated after 24 hours of culture. The panel presents the means ± S. E. M. (n= 3).



Supplementary Figure S9. The fitness of *C. albicans* is affected by pyocyanin (10 µg/ml). The fitness (m) was determined based on the CFU/ml values obtained after 24 hours of culture. The panel presents the means ± S. E. M. (n= 3).



Supplementary Figure S10. Survival of mice infected with *C. albicans* (CA), *P. aeruginosa* (PA) or coinfected with *C. albicans*/P. aeruginosa (Mix). Results are from 6 mice per group.

Supplementary Tables S1-S5.

Supplementary Table S1. Functional classification of *P. aeruginosa* identified proteins that were differentially expressed in mixed biofilms.

ORF[a]	Protein	Description	Score[b]	MS sequence coverage %	Fold change[d]	t-test *P* value
		Pyoverdine synthesis				
PA2386	PvdA	L-ornithine N5-oxygenase	270	62	3.62	0.037
PA2386*	PvdA	L-ornithine N5-oxygenase	215	59	481.42	0.014
PA2386*	PvdA	L-ornithine N5-oxygenase	106	38	1.62	0.043
PA2396	PvdF	Pyoverdine synthetase F	94	16	1.87	0.009
PA2413	PvdH	L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase	161	53	2.13	0.032
		Siderophore transport				
PA4221	FptA	Fe(III)-pyochelin outer membrane receptor precursor	268	63	3.44	0.006
PA4221*	FptA	Fe(III)-pyochelin outer membrane receptor precursor	231	44	3.40	0.045
PA4221*	FptA	Fe(III)-pyochelin outer membrane receptor precursor	250	44	3.44	0.034
PA4221*	FptA	Fe(III)-pyochelin outer membrane receptor precursor	179	35	3.17	0.019
PA4221*	FptA	Fe(III)-pyochelin outer membrane receptor precursor	160	34	0.58	0.044
PA2688	PfeA	Ferric enterobactin receptor	138	31	3.00	0.007
PA4168	FpvB	Second ferric pyoverdine receptor FpvB	172	26	2.28	0.021
PA4168*	FpvB	Second ferric pyoverdine receptor FpvB	109	19	1.67	0.013
PA4675	ChtA	Probable TonB-dependent receptor	107	17	1.68	0.04
		Heme biosynthesis and transport				
PA5243	HemB	Delta-aminolevulinic acid dehydratase	129	41	0.59	0.042
PA3408	HasR	Heme uptake outer membrane receptor	201	22	2.00	0.011
		Proteases				
PA4370	IcmP	Insulin-cleaving metalloproteinase	164	47	2.85	0.039
PA0766	MucD	Serine protease	165	50	2.61	0.031
		Motility & Attachment				
PA5040	PilQ	Type 4 fimbrial biogenesis outer membrane protein	267	55	1.88	0.023
PA5040*	PilQ	Type 4 fimbrial biogenesis outer membrane protein	247	45	1.76	0.031
PA5040*	PilQ	Type 4 fimbrial biogenesis outer membrane protein	228	44	1.52	0.007

PA3105	XcpQ	General secretion pathway protein D	101	27	2.28	0.019
		Oxidative stress protection				
PA4236	KatA	Catalase	68	30	0.34	0.026
PA3529	PA3529	Probable peroxidase	99	44	0.23	0.018
PA0139	AhpC	Alkyl hydroperoxide reductase subunit C	98	54	0.01	0.013
PA0139*	AhpC	Alkyl hydroperoxide reductase subunit C	97	44	0.52	0.031
PA0139*	AhpC	Alkyl hydroperoxide reductase subunit C	89	36	0.52	0.015
PA0407	GshB	Glutathione synthetase	147	60	0.00	0.04
		Protein folding				
PA4385	GroEL	Chaperonin HSP70	84	18	1.46	0.015
PA1805	PpiD	Peptidyl-prolyl cis-trans isomerase D	326	66	2.06	0.036
		Transport of small molecules and antibiotic resistance				
PA3790	OprC	Putative copper transport outer membrane porin	157	34	2.62	0.017
PA3790*	OprC	Putative copper transport outer membrane porin	97	37	1.97	0.019
PA3648	Opr86	Outer membrane protein	157	24	1.76	0.026
PA3186	OprB	Glucose/carbohydrate outer membrane porin	107	30	0.12	0.032
PA3186*	OprB	Glucose/carbohydrate outer membrane porin	105	37	0.39	0.021
PA0291	OprE	Anaerobically-induced outer membrane porin	206	55	1.68	0.008
PA1777	OprF	Major porin and structural outer membrane porin	98	48	1.69	0.003
PA1777*	OprF	Major porin and structural outer membrane porin	114	43	1.52	0.031
PA0973	OprL	Peptidoglycan associated lipoprotein	89	52	1.78	0.027
PA2760	OprQ	Outer membrane porin	167	52	1.93	0.012
PA2113	OpdO	Pyroglutamate porin	248	60	3.21	0.005
PA4501	OpdP	Glycine-glutamate dipeptide porin	154	44	1.57	0.009
PA4974	OpmH	Probable outer membrane protein	324	82	2.34	0.02
PA4461	PA4461	Probable ATP-binding component of ABC transporter	175	62	0.52	0.036
PA1260	PA1260	Amino acid ABC transporter	103	31	0.06	0.007
		Cell wall / LPS / capsule				
PA3159	WbpA	UDP-N-acetyl-d-glucosamine 6-Dehydrogenase	163	53	2.49	0.013
PA5163	RmlA	Glucose-1-phosphate thymidylyltransferase	104	35	1.65	0.031
PA5552	GlmU	Glucosamine-1-phosphate acetyltransferase/N-acetylglucosamine-1-phosphate uridyltransferase	111	42	1.59	0.003

PA5549	GlmS	Glucosamine-fructose-6-phosphate aminotransferase	117	19	0.01	0.004
PA3636	KdsA	2-dehydro-3-deoxyphosphooctonate aldolase	111	52	0.51	0.042
PA5078	OpgG	Glucan biosynthesis protein G	182	38	0.44	0.003
		Glycolysis / Gluconeogenesis				
PA2323	PA2323	Probable glyceraldehyde-3-phosphate dehydrogenase	104	29	1.33	0.027
PA1770	PpsA	Phosphoenolpyruvate synthase	97	14	0.31	0.001
PA0555	FdA	Fructose-1,6-bisphosphate aldolase	100	26	0.23	0.042
PA5192	PckA	Phosphoenolpyruvate carboxykinase	96	24	0.06	0.012
PA5015	AceE	Pyruvate dehydrogenase	81	24	0.17	0.004
PA5015	AceE	Pyruvate dehydrogenase	130	37	0.53	0.023
		Tricarboxylic acid cycle				
PA0795	PrpC	Citrate synthase 2	211	49	0.56	0.036
PA1562	AcnA	Aconitate hydratase 1	221	37	0.44	0.008
PA2624	IdH	Isocitrate dehydrogenase	110	20	0.37	0.019
PA1585	SucA	2-oxoglutarate dehydrogenase (E1 subunit)	203	34	2.30	0.021
PA1585*	SucA	2-oxoglutarate dehydrogenase (E1 subunit)	110	41	1.75	0.031
PA1586	SucB	Dihydrolipoamide succinyltransferase (E2 subunit)	112	42	2.04	0.014
PA1588	SucC	Succinyl-CoA synthetase beta chain	117	46	0.31	0.007
PA1589	SucD	Succinyl-CoA synthetase alpha chain	114	43	0.12	0.008
PA1583	SdhA	Succinate dehydrogenase (A subunit)	98	23	0.37	0.041
PA0854	FumC2	Fumarate hydratase	141	40	0.32	0.011
PA0854*	FumC2	Fumarate hydratase	111	34	0.47	0.005
		Energy metabolism				
PA4031	PpA	Inorganic pyrophosphatase	91	50	0.01	0.006
PA5556	AtpA	ATP synthase alpha chain	174	50	0.56	0.031
PA5556*	AtpA	ATP synthase alpha chain	169	43	0.09	0.012
PA5557	AtpH	ATP synthase delta chain	101	53	1.60	0.009
		Transcription				
PA0002	DnaN	DNA polimerasa III	85	26	0.23	0.041
PA3168	GyrA	DNA gyrase subunit A	112	21	0.08	0.032
PA4275	NusG	Antiterminador de la transcripcion	120	51	0.43	0.013
		Translation				
PA4265	TufA	Factor de elongacion Tu	125	34	0.42	0.008
PA4671	RplY	Probable ribosomal protein L25	86	66	0.31	0.019
PA4266	FusA1	Elongation factor G	83	24	0.15	0.009
PA3655	TsF	Elongation factor Ts	150	61	0.01	0.021
PA3653	Frr	Ribosome recycling factor	87	51	0.01	0.042
PA3653*	Frr	Ribosome recycling factor	132	60	0.02	0.033

PA4740	PnP	Polyribonucleotide nucleotidyltransferase	100	12	1.65	0.041
PA0592	KsgA	rRNA (adenine-N6,N6)-dimethyltransferase	107	33	0.21	0.034
PA0963	AspS	Aspartyl-tRNA synthetase	96	16	0.10	0.029
PA0956	ProS	Prolyl-tRNA synthetase	175	47	0.31	0.017
PA0956*	ProS	Prolyl-tRNA synthetase	113	35	0.38	0.023
		Amino acid biosynthesis and metabolism				
PA4696	IlvI	Acetolactate synthase large subunit	90	21	1.14	0.009
PA4694	IlvC	Ketol-acid reductoisomerase	84	32	5.12	0.019
PA5013	IlvE	Branched-chain amino acid transferase	107	50	0.59	0.021
PA3082	GbT	Glycine betaine transmethylase	94	28	2.09	0.041
PA3082*	GbT	Glycine betaine transmethylase	82	19	4.23	0.039
PA4759	DapB	Dihydrodipicolinate reductase	119	68	0.56	0.023
PA1254	PA1254	Probable dihydrodipicolinate synthetase	106	31	0.27	0.017
PA0035	TrpA	Tryptophan synthase alpha chain	87	23	0.01	0.019
PA1587	lpdG	Lipoamide dehydrogenase-glc	88	19	0.37	0.021
PA0447	GcdH	Glutaryl-CoA dehydrogenase	101	49	0.32	0.027
		Nucleotide biosynthesis and metabolism				
PA3686	AdK	Adenylate kinase	113	49	0.02	0.032
PA0143	NuH	Purine nucleosidase	130	62	1.60	0.041
PA3970	AmN	AMP nucleosidase	127	31	0.52	0.008
PA3004	MtnP	5-methylthioadenosine phosphorylase	87	37	1.54	0.013
PA3770	GuaB	Inosine-5'-monophosphate dehydrogenase	132	77	1.56	0.025
		Hypothetical/probable				
PA4390	PA4390	Hypothetical protein	69	17	2.43	0.006
PA0537	PA0537	Hypothetical protein	86	40	0.45	0.043
PA0079	PA0079	Hypothetical protein	139	43	0.32	0.041
PA0588	PA0588	Hypothetical protein	128	15	0.09	0.008
PA0588*	PA0588	Hypothetical protein	103	18	0.07	0.019
PA1202	PA1202	Probable hydrolase	86	35	0.42	0.037
PA1344	PA1344	Probable short-chain dehydrogenase	107	34	0.01	0.012

[a] Pseudomonas Genome database.

[b] MASCOT score.

[c] Change in expression between mixed biofilm and monoespecific biofilm.

*Proteins show both a shift in charge and mass with respect to the theoretical values.

Supplementary Table S2. Functional classification of *C. albicans* identified proteins that were differentially expressed in mixed biofilms.

CGD[a] Systematic Name	Protein	Description	Hap43p regulated[b]	Score[c]	MS Sequence coverage %	Fold Change[d]	t-test *P* value
		Glycolysis					
orf19.542	Hxk2p	Hexokinase II		100	24	134	0.034
orf19.6745	Tpi1p	Triose-phosphate isomerase		92	39	0.65	0.015
orf19.6745*	Tpi1p	Triose-phosphate isomerase		72	27	0.62	0.04
orf19.6814	Tdh3p	Glyceraldehyde-3-phosphate dehydrogenase		142	31	0.47	0.007
orf19.3651	Pgk1p	Phosphoglycerate kinase		94	26	0.52	0.003
orf19.903	Gpm1p	Phosphoglycerate mutase	Hap43p	99	43	0.55	0.017
orf19.3575	Cdc19p	Pyruvate kinase	Hap43p	97	43	0.61	0.027
		Glucose uptake					
orf19.13	orf19.13	Putative glucokinase		85	17	2.24	0.042
		Gluconeogenesis					
Orf19.3419	Mae1p	Malic enzyme	Hap43p	65	11	0.51	0.019
		Pyruvate dehydrogenase complex					
orf19.6127	Lpd1p	Putative dihydrolipoamide dehydrogenase		111	30	0.65	0.015
orf19.3097	Pda1p	Putative pyruvate dehydrogenase alpha chain		88	26	0.27	0.027
orf19.3097*	Pda1p	Putative pyruvate dehydrogenase alpha chain	Hap43p	72	17	0.63	0.012
		Tricarboxylic acid cycle					
orf19.6385	Aco1p	Aconitase	Hap43p	93	12	0.51	0.009
orf19.5791	Idh2p	Protein described as isocitrate dehydrogenase subunit		95	35	0.56	0.02
orf19.5791*	Idh2p	Protein described as isocitrate dehydrogenase subunit		82	28	0.66	0.036
orf19.4602	Mdh1-1p	Predicted malate dehydrogenase		96	29	0.41	0.007
		Fermentation					
orf19.2877	Pdc11p	Pyruvate decarboxylase	Hap43p	97	32	0.46	0.013
orf19.2877*	Pdc11p	Pyruvate decarboxylase	Hap43p	93	17	0.58	0.031

orf19.5806	Ald5p	NAD-aldehyde dehydrogenase		101	20	2.55	0.008
orf19.5806*	Ald5p	NAD-aldehyde dehydrogenase		90	20	1.82	0.019
		Aerobic respriation					
orf19.2644	Qcr2	Ubiquinol-cytochrome-c reductase	Hap43p	132	35	0.48	0.021
		Formate oxidation					
orf19.638	Fdh1p	Formate dehydrogenase		124	43	2.31	0.033
orf19.638*	Fdh1p	Formate dehydrogenase		124	43	2.18	0.041
		Acetate utilization					
orf19.3171	Ach1p	Acetyl-coA hydrolase		96	28	0.64	0.029
		Heme biosynthesis					
orf19.2803	Hem13p	Coproporphyrinogen III oxidase	Hap43p	143	35	0.56	0.007
orf19.2803*	Hem13p	Coproporphyrinogen III oxidase	Hap43p	97	23	0.58	0.031
orf19.1880	Hem15p	Putative ferrochelatase involved in heme biosynthesis		84	18	0.43	0.013
		Pentose phosphate pathway					
orf19.4618	Fba1p	Fructose-bisphosphate aldolase		77	29	2.03	0.003
orf19.4618*	Fba1p	Fructose-bisphosphate aldolase		115	30	1.51	0.017
orf19.5024	Gnd1p	6-phosphogluconate dehydrogenase		72	14	11.4	0.009
orf19.5024*	Gnd1p	6-phosphogluconate dehydrogenase		72	14	1.84	0.034
orf19.5024*	Gnd1p	6-phosphogluconate dehydrogenase		101	19	2.1	0.041
orf19.704	Sol3p	Predicted ORF		161	51	1.82	0.008
orf19.5112	Tkl1p	Putative transketolase		78	17	1.65	0.016
orf19.4754	Zwf1p	Glucose-6-phosphate dehydrogenase		97	19	1.5	0.021
orf19.4754*	Zwf1p	Glucose-6-phosphate dehydrogenase		97	19	1.53	0.043
orf19.4754*	Zwf1p	Glucose-6-phosphate dehydrogenase		97	19	1.61	0.007
orf19.4754*	Zwf1p	Glucose-6-phosphate dehydrogenase		97	19	3.09	0.018

orf19.4754*	Zwf1p	Glucose-6-phosphate dehydrogenase		111	23	2.92	0.024
		Oxidative stress protection					
orf19.6229	Cat1p	Catalase	Hap43p	91	28	0.32	0.036
orf19.5180	Prx1p	Thioredoxin peroxidase		71	31	0.52	0.008
orf19.2241	Pst1p	Putative 1,4-benzoquinone reductase	Hap43p	72	17	0.65	0.012
orf19.113	Cip1p	Possible oxidoreductase		155	44	0.24	0.029
orf19.7417	Tsa1p	TSA/alkyl hydroperoxide peroxidase C		82	35	4.2	0.014
orf19.7417*	Tsa1p	TSA/alkyl hydroperoxide peroxidase C		92	38	2.64	0.009
Orf19.2762	Ahp1p	Putative alkyl hydroperoxide reductase	Hap43p	92	38	4.75	0.019
		Stress response					
orf19.2013	Kar2p	Similar to chaperones of Hsp70p family		73	18	0.63	0.021
orf19.2013*	Kar2p	Similar to chaperones of Hsp70p family		152	27	0.42	0.042
orf19.4980	Hsp70p	Putative hsp70 chaperone		98	21	2.52	0.007
orf19.4980*	Hsp70p	Putative hsp70 chaperone		88	32	1.76	0.036
orf19.882	Hsp78p	Putative heat-shock protein		96	18	1.68	0.025
orf19.1065	Ssa2p	HSP70 family chaperone		92	37	0.51	0.009
orf19.6367	Ssb1p	HSP70 family heat shock protein	Hap43p	87	16	0.53	0.011
orf19.6367*	Ssb1p	HSP70 family heat shock protein	Hap43p	98	19	0.65	0.038
orf19.6367*	Ssb1p	HSP70 family heat shock protein	Hap43p	93	16	1.41	0.018
Orf19.6367*	Ssb1p	HSP70 family heat shock protein	Hap43p	71	16	0.47	0.036
orf19.3812	Ssz1p	Putative HSP70 chaperone		112	27	0.51	0.013
orf19.1552	Cpr3p	Putative peptidyl-prolyl cis-trans isomerase		95	47	5.7	0.005
orf19.4777	Dak2p	Putative dihydroxyacetone kinase		119	22	1.5	0.012
orf19.2613	Ecm4p	Cytoplasmic glutathione S-transferase		111	39	2.03	0.041
orf19.3150	Gre2p	Methylglyoxal reductase		109	31	1.54	0.031
orf19.4309*	Grp2p	Methylglyoxal reductase	Hap43p	237	60	1.57	0.027
orf19.4309*	Grp2p	Methylglyoxal reductase	Hap43p	147	41	0.41	0.012

orf19.4371	Tal1p	Transaldolase	185	55	5.05	0.026
		Amino acids metabolism				
Orf19.1613	Ilv2p	Putative acetolactate synthase	72	11	2.12	0.013
orf19.2525	Lys12p	Putative mitochondrial homoisocitrate dehydrogenase	200	48	1.6	0.031
orf19.7448	Lys9p	Putative enzyme of lysine biosynthesis	130	34	4.2	0.015
orf19.2551	Met6p	cobalamin-independent methionine synthase	193	29	2.51	0.04
orf19.2551*	Met6p	cobalamin-independent methionine synthase	140	25	2.13	0.043
orf19.657	Sam2p	S-adenosylmethionine synthetase	78	27	0.45	0.009
orf19.657*	Sam2p	S-adenosylmethionine synthetase	106	27	0.5	0.032
orf19.657*	Sam2p	S-adenosylmethionine synthetase	97	29	1.6	0.013
orf19.4718	Trp5p	Predicted tryptophan synthase	111	18	1.9	0.005
orf19.2951	Hom6p	Putative homoserine dehydrogenase	85	16	4.3	0.012
orf19.1153	Gad1p	Putative glutamate decarboxylase	91	20	1.8	0.041
orf19.4716	Gdh3p	NADP-glutamate dehydrogenase	130	53	1.7	0.031
		Translation				
orf19.5788	Eft2p	Elongation Factor 2	112	17	0.43	0.012
orf19.7626	Eif4ep	Translation initiation factor eIF4E	101	33	0.62	0.013
orf19.437	Grs1p	Putative tRNA-Gly synthetase	89	27	0.65	0.043
orf19.6749	Krs1p	Putative tRNA-Lys synthetase	94	24	0.58	0.037
Orf19.269p	Ses1p	Seryl-tRNA synthetase	84	27	0.41	0.042
Orf19.269p*	Ses1p	Seryl-tRNA synthetase	70	17	0.55	0.011
		Nucleotides metabolism				
orf19.492	Ade17p	5-Aminoimidazole-4-carboxamide ribotide transformylase	170	35	0.62	0.039
orf19.5906	Ade2p	Phosphoribosylaminoimadazole carboxylase	123	24	1.6	0.031

orf19.5591	Ado1p	Adenosine kinase	158	43	1.5	0.019
orf19.18	Imh3p	Inosine monophosphate (IMP) dehydrogenase	89	23	3.0	0.023
		Lipids metabolism				
orf19.1064	Acs2p	Probable acetyl-CoA synthetase	186	30	2.1	0.007
orf19.1591	Erg10p	Acetyl-CoA acetyltransferase	133	29	1.9	0.041
orf19.1591*	Erg10p	Acetyl-CoA acetyltransferase	94	23	1.2	0.016
orf19.1155	Dpp2	Putative dihydrolipoamide dehydrogenase	85	17	3.1	0.011
		Virulence				
orf19.5197	Ape2p	Neutral arginine, alanine, leucine specific metallo-aminopeptidase	80	10	2.2	0.032
orf19.5197*	Ape2p	Neutral arginine, alanine, leucine specific metallo-aminopeptidase	102	14	2.8	0.026
orf19.4565	Bgl2p	1,3-beta-Glucosyltransferase	80	30	1.5	0.031
orf19.1680	Tfp1p	Subunit of vacuolar H+-ATPase	75	15	1.6	0.019
		Filamentation				
orf19.5968	Rdi1p	Protein similar to S. cerevisiae Rdi1p	182	68	0.53	0.026
orf19.3014	Bmh1p	Sole 14-3-3 protein in C. albicans	97	36	1.6	0.032
		Xylose metabolism				
orf19.4317	Gre3p	Putative D-xylose reductase	74	18	1.7	0.008
orf19.7676	Xyl2p	Protein described as similar to D-xylulose reductase	93	23	2.0	0.003
orf19.7676*	Xyl2p	Protein described as similar to D-xylulose reductase	74	21	1.5	0.044
orf19.7676*	Xyl2p	Protein described as similar to D-xylulose reductase	83	26	2.5	0.007
orf19.7676*	Xyl2p	Protein described as similar to D-xylulose reductase	98	30	1.9	0.027
		Uncharacterized proteins				
orf19.2966	orf19.2966p	Predicted ORF	109	24	4.0	0.04
orf19.3053	orf19.3053	Protein of unknown function	74	32	1.6	0.026
orf19.3508	orf19.3508p	Predicted ORF	85	24	1.6	0.018

Orf19.3932	Orf19.3932p	Stationary phase enriched protein		133	40	0.61	0.013
Orf19.5620	Orf19.5620p	Stationary phase enriched protein		92	28	2.6	0.031
orf19.590	orf19.590p	Predicted ORF		123	24	1.4	0.015
orf19.6809	orf19.6809	Putative phosphomutase-like protein	Hap43p	85	26	0.38	0.04
orf19.7261	orf19.7261	Uncharacterized		81	23	11.4	0.042
orf19.7310	orf19.7310	Protein with a role in directing meiotic recombination events	Hap43p	80	12	0.66	0.037

[a] CGD, Candida Genome Database

[b] Proteins whose genes were previously shown to be Hap43p-regulated (Hsu *et al*., 2011).

[c] MASCOT score.

[d] Change in expression between mixed biofilm and monoespecific biofilm.

*Proteins show both a shift in charge and mass with respect to the theoretical values.

Supplementary Table S3. Extracellular proteins from *P. aeruginosa* that were differentially expressed in mixed biofilms.

Systematic name[a]	**Protein**	**Description**	**Score[b]**	**MS Sequence coverage**	**Fold change[c]**	**t-test *P* value**
		Exotoxin				
PA1148	ToxA	Exotoxin A precursor	211	30	2.27	0.018
PA1148	ToxA	Exotoxin A precursor	235	34	6.89	0.031
		Proteases				
PA1249	AprA	Alkaline metalloproteinase	67	16	1.70	0.042
PA0423	PasP	Protease	123	47	2.58	0.017
PA4175	PrpL	Protease IV	82	20	1.54	0.038
PA4175	PrpL	Protease IV	75	18	1.89	0.011
PA0572	PA0572	Hypothetical protein zinc-protease	103	19	8.40	0.007
PA0572	PA0572	Hypothetical protein zinc-protease	121	21	2.85	0.005
PA0572	PA0572	Hypothetical protein zinc-protease*	133	14	1.80	0.013
PA0572	PA0572	Hypothetical protein zinc-protease*	130	23	1.53	0.004
PA0572	PA0572	Hypothetical protein zinc-protease*	226	26	1.66	0.016
PA0572	PA0572	Hypothetical protein zinc-protease*	136	23	1.81	0.021
PA0572	PA0572	Hypothetical protein zinc-protease*	109	22	3.00	0.009

PA0572	PA0572	Hypothetical protein zinc-protease*	172	22	3.94	0.019
PA0572	PA0572	Hypothetical protein zinc-protease*	153	19	5.42	0.012
PA0572	PA0572	Hypothetical protein zinc-protease*	159	24	6.68	0.028
PA0572	PA0572	Hypothetical protein zinc-protease*	107	18	5.54	0.031
PA0572	PA0572	Hypothetical protein zinc-protease*	103	21	10.66	0.026
PA0572	PA0572	Hypothetical protein zinc-protease*	116	17	6.32	0.013
PA0572	PA0572	Hypothetical protein zinc-protease*	162	21	5.00	0.008
PA0572	PA0572	Hypothetical protein zinc-protease*	100	20	6.36	0.005
PA3724	LasB	Elastase LasB*	86	28	0.37	0.016
PA3724	LasB	Elastase LasB*	85	31	1.53	0.021
PA3724	LasB	Elastase LasB*	89	26	0.70	0.018
PA3724	LasB	Elastase LasB*	88	22	0.63	0.009
PA3724	LasB	Elastase LasB*	87	18	0.36	0.036
PA2939	PepB	Leucine-specific aminopeptidase	68	17	0.27	0.047
		Secreted Factors				
PA0852	CbpD	Chitin-binding protein	90	23	0.54	0.019
PA0852	CbpD	Chitin-binding protein*	93	24	0.48	0.035
PA0852	CbpD	Chitin-binding protein *	85	22	0.20	0.023
PA0852	CbpD	Chitin-binding protein *	89	25	0.40	0.041
		Siderophore biosynthesis and transport				
PA2385	PvdQ	3-oxo-C12-homoserine lactone acylase PvdQ	165	25	2.42	0.037
PA2386	PvdA	L-ornithine N5-oxygenase	261	51	1.95	0.009
PA2398	FpvA	Ferripyoverdine receptor	231	39	12.78	0.016
PA2398	FpvA	Ferripyoverdine receptor	187	25	2.55	0.013
PA2413	PvdH	L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase, PvdH	165	27	2.86	0.007
PA4221	FptA	Fe(III)-pyochelin outer membrane receptor precursor	317	50	3.20	0.032
PA4228	PchD	Pyochelin biosynthesis protein	115	24	3.37	0.023
PA2451	PA2451	Hypothetical protein, Enterochelin esterase	99	26	1.98	0.009
PA2452	PA2452	Hypothetical protein	209	59	2.37	0.018
PA2452	PA2452	Hypothetical protein	165	39	2.93	0.046
		Heme uptake				
PA3407	HasA	Hemophore protein	82	19	1.97	0.033
PA3408	HasR	Heme uptake outer membrane receptor	205	28	2.22	0.041
		Membrane proteins				
PA1777	OprF	Outer membrane porin	92	36	2.59	0.036
PA0958	OprD	Outer membrane porin	122	25	1.69	0.042

PA4837	PA4837	Probable outer membrane protein precursor	376	62	1.76	0.018
PA4974	PA4974	Probable outer membrane protein precursor	120	38	1.52	0.021
		Flagellar filament protein				
PA1092	FliC	Flagellin type B	160	39	2.47	0.009
		Translation				
PA4266	FusA1	Elongation factor G	82	16	0.43	0.048
		RNA processing and degradation				
PA4740	PnP	Polyribonucleotide nucleotidyltransferase	100	21	0.55	0.032
		Purine nucleotide metabolic process				
PA3770	GuaB	Inosine-5'-monophosphate dehydrogenase	129	24	2.65	0.046
		Fatty acid and phospholipid metabolism				
PA4848	AccC	Biotin carboxylase	86	16	1.64	0.035
		Pyruvate metabolism				
PA5015	AceE	Pyruvate dehydrogenase complex component E1	97	17	0.42	0.027
		Oxidative phosphorylation				
PA5554	AtpD	ATP synthase beta chain	156	45	0.38	0.048
PA5556	AtpA	ATP synthase alpha chain	152	27	0.45	0.039

[a] Pseudomonas Genome database.

[b] MASCOT score.

[c] Change in expression between mixed biofilm and monoespecific biofilm.

[d] *P*-value was obtained by Student test.

*Spots corresponded to cleavage fragments of a larger translated ORF.

Supplementary Table S4. Characteristics of 30 individual Rif^R mutants randomly isolated from 24 h old cocultures. Mutants with identical characteristics are marked with an asterisk. Method for DNA sequence analysis to identify Rif^R mutations is described in Supplementary Methods.

Isolate	*rpoB* mutation[a]	Tc sensitivity[b]	Cm sensitivity[b]	Pyocyanin[c]	Pellicle morphology[d]	Cell aggregation[e]
wild type	None	S	S	100	S	-
A1	1736 C>A	S	S	110	S	-
A2	1562 A>T	S	S	178	S	-
A3	1592 A>T	S	S	43	S	+
A4	1550 C>T	R	S	95	S	-
A5	1580 C>T	S	S	123	S	-
A6	1549 T>C	R	S	169	W	-
A7	1607 C>T	S	S	87	S	-
A8	1562 A>T	S	R	117	S	-
A9	1580 C>T	S	S	103	S	+
A10	1562 A>G	S	S	215	S	-
A11	1592 A>G	R	R	155	S	-
A12	1562 A>G	S	S	97	W	-
B1	1549 T>C	S	R	104	S	-
B2*	1562 A>G	S	S	89	S	-
B3	1553 A>T	S	S	108	S	+
B4	1562 A>G	S	S	121	W	-
B5	1736 C>A	R	S	105	S	-
B6*	1562 A>G	S	S	100	S	-

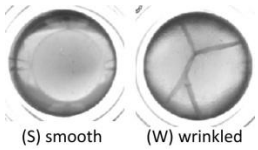
B7	1592 A>T	S	S	275	S	-
B8	1906 G>T	S	S	187	S	+
B9*	1562 A>G	S	S	106	S	-
B10	1706 C>T	S	S	112	S	-
B11	1562 A>G	S	R	104	S	-
B12	1542 C>G	S	S	108	S	-
C1	1906 G>T	S	S	54	S	-
C2	1613 T>C	S	R	123	S	-
C3	1562 A>G	R	R	281	S	-
C4	1540 A>C	S	S	91	S	+
C5	1562 A>G	S	S	112	W	-
C6	1592 A>T	R	S	98	S	+

[a]Numbering of sequences is from the first nucleotide in the start codon of *rpoB*.

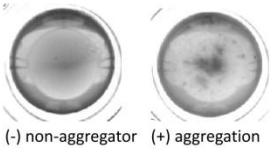
[b]Sensitivity to tetracycline (Tc, 15 µg/ml) or *chloramphenicol* (Cm, 100 µg/ml) is shown as the growth (**R**) or not growth (**S**) at 16 h in liquid LB medium plus antibiotic. The medium was inoculated at OD_{600nm} of 0.01. Growth was measured by optical density from two independent cultures of each isolated.

[c]The pyocyanin production values are presented as a percentage relative to the wild type. To determine the pyocyanin pigment, the wild-type and Rif^R mutants were grown for 24 h at 37 C without shaking in fetal bovine serum (SFB). The pyocyanin quantification was performed using chloroform extraction as described by Cugini *et al*. (2007).

[d]A ‘pellicle’ is a biofilm formed at the air–liquid interface of a standing culture. (S) indicates smooth pellicle formation and (W) indicates wrinkled pellicle formation in FSB at 24 h of growth without shaking at 37 C.



[e]Cell aggregation was evaluated visually in SFB at 24 h of growth without shaking at 37 C. Symbols (-) indicates non-aggregator and (+) indicates aggregation as is exemplified by the following pictures:



Supplementary Table S5. Amino acid concentrations (µM ± SD) in the supernatants of cultures after 24 hours of culture.

	P. aeruginosa	*C. albicans*	*Coculture*	RPMI
Glutamine	179.43 ± 41.75	229.76 ± 55.56	19.93 ± 13.4	2000
Aspartate	64.65 ± 22.06	186.33 ± 66.53	16.58 ± 14.12	379
Arginine	55.47 ± 18.38	288.8 ± 57.75	9.26 ± 9.88	1150

Supplementary Materials and Methods

Separation of bacterial and fungal cells

The cells were separated through discontinuous Percoll gradient centrifugation (Percoll; Sigma Aldrich, P1644). Seven stock solutions of 50, 60, 65 and 70% Percoll were prepared in PBS, and 2.5 ml of each solution was layered manually. Biofilms were harvested and sonicated for 10 seconds in 2 ml of ice-cold stop buffer and then layered on the top of the gradients, which were centrifuged at 300 ×*g* for 5 min and 8,000 ×*g*

for 10 min using a fixed-angle rotor at 5 °C. The top (*P. aeruginosa*) and lower (*C. albicans*) fractions were collected and washed twice with 5 volumes of stop buffer.

Purification of whole-cell proteins

Proteins were obtained via sonication at 24 kHz for 30 s in the on position and 1 min in the off position for five cycles at 4 °C using a Soniprep 150 sonicator (MSE, UK) in the presence of a protease inhibitor (Complete tablets; Roche Diagnostics GmbH, Mannheim, Germany). To further limit proteolysis, protein isolation was performed through phenol extraction (Hurkman & Tanaka, 1986). To solubilize proteins and to obtain completely denatured and reduced proteins, the pellets were dried and resuspended as previously described (Encarnación *et al*., 2003). Prior to electrophoresis, the samples were mixed with 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (Roche Diagnostics GmbH, Germany), 2 mM tributyl phosphine, 2% ampholytes and 60 mM dithiothreitol.

Protein Identification via MALDI-TOF MS

Selected spots from Coomassie blue-stained preparative two-dimensional gels were excised manually and frozen at –70 °C until being used. The samples were prepared for mass spectrum analysis by applying a slight modification to a previously described procedure (Encarnación *et al*., 2003). The protein spots were destained, reduced, alkylated and digested with trypsin (Promega, Madison, WI). The peptide mixtures were desalted using a C_{18} Zip Tip (Millipore, Bedford, MA), according to the manufacturer's recommendations.

Mass spectra were obtained using a MALDI-TOF mass spectrometer autoflex™ (Bruker Daltonics, Billerica, MA) operated in delayed extraction and reflectron mode. The spectra were externally calibrated using a peptide calibration standard (Bruker Daltonics 206095). Peptide mixtures were analyzed using a saturated solution of alpha-cyano-4-hydroxycinnamic acid in 50% acetonitrile-0.1% trifluoroacetic acid. The resulting monoisotopic list of *m*/*z* values was submitted to the search engine MASCOT (Perkins *et al.*, 1999), searching all protein and DNA sequence information available from public databases (Swiss-Prot and NCBInr). The following search parameters were applied: mass accuracy, ± 0.35 Da; fixed modification, carbamidomethylation; variable modification, methionine oxidation; and a maximum of one tryptic missed cleavage. A protein was considered to be identified if the scores from the database searches clearly exceeded the algorithm's significance threshold ($P<0.05$).

Detection and quantification of metabolites

Detection of AHLs was conducted following the methodology of Shau *et al.* (1997), with slight modifications (Supplementary Information). For pyoverdine quantification, cells were removed via centrifugation, and the absorbance of the supernatant at 405 nm was determined (Hohnadel *et al.*, 1986). The concentrations were calculated based on the absorbance values for purified standards (Pyoverdines, Cat. No. P8124, Sigma-Aldrich) dissolved in RPMI medium. The obtained A_{405} value was divided by the OD_{600} of the culture to indicate the amount of pyoverdine produced per cell.

Rhamnolipids were extracted from the supernatant and detected via TLC as previously described (Caiazza *et al.*, 2005). The TLC plate was scanned with a GS-800 imaging densitometer (Bio-Rad), and fold changes in spot intensity were determined with PDQuest 2-D Analysis software (Bio-Rad). The concentration of D-glucose in the culture supernatant was determined through an enzymatic assay using a D-glucose test kit (Cat. No. 10 716 251 035, R-BIOPHARM, ROCHE).
Pyocyanin was extracted, and the concentration in the culture supernatant was determined via HPLC. At the specified time points, the supernatant was removed and passed through a 0.22-μm filter. Then, a 5 ml aliquot of the cleared supernatant was mixed with 2.5 ml of chloroform, and the samples were vortexed for 30 s and centrifuged for 2 min, after which 1 ml of the lower chloroform layer was removed. A second extraction of the same 5 ml aliquot was performed, and

the chloroform layer was collected and combined with the first extract. The extracts were resuspended in 50 µl of methanol, and the pyocyanin concentration was determined through HPLC analysis in a Waters Symmetry C18 reverse-phase column. The solvent system consisted of 0.05% trifluoroacetic acid (TFA) in water and 0.05% TFA in acetonitrile, and 25 to 30 min runs were conducted. Calculations were performed based on absorbance values for purified standards (Pyocyanin, Cat. No. P0046, Sigma-Aldrich).

Confocal laser scanning microscopy

The biofilms formed on polystyrene slides were analyzed via confocal laser scanning microscopy. The slides were constructed from polystyrene plates, sterilized for 30 minutes with ultraviolet light and immersed in Petri plates under culture conditions identical to those described for obtaining biofilms for proteome analysis. Following biofilm formation, the slides were removed and transferred to 50-ml conical tubes and washed once with fresh medium. To monitor the viability of cells, biofilms were stained using the BacLight LIVE/DEAD staining system according to the manufacturer's protocol (#L7007, Molecular Probes). The biofilms were stained for 30 minutes, washed once with fresh medium and observed with a Zeiss LSM 510 META confocal laser scanning microscope equipped with 488-nm, 514-nm, 543-nm and 633-nm laser lines.

Enzymatic assay for catalase activity

A standard enzymatic assay (Hassett *et al*., 1999) was used to measure total catalase levels for *C. albicans and P. aeruginosa*. Cells were disrupted via sonication in potassium phosphate buffer, followed by centrifugation to obtain cell extracts. A total of 40 µg of protein was used in the assays, and the recorded enzymatic activity was standardized for protein contents.

Iron and oxygen quantitation

The concentration of iron ions in the culture supernatant was determined separately for Fe^{2+} and Fe^{3+} through spectrophotometric assays using iron test kits (Merck). The concentration of dissolved oxygen in the culture was determined using an OXEL-1 oxygen electrode (WPI).

Determination of mutation frequencies associated with resistance to antibiotics and fungicides

After 24 hours of cultivation, single-species biofilms and mixed biofilms were scraped from the culture plates and disaggregated in 20 ml of Tween solution (10 mM $MgSO_4$ and 20 mM Tween 20). Then, 1 ml of the biofilm suspension was inoculated into 10 ml of LB medium to recover the bacterium or into 10 ml of YPD (10 µg/ml chloramphenicol was used to reduce the growth of bacteria) medium to recover the fungus. The cultures were incubated for 12 hours at 37 °C and shaken at 250 rpm. As appropriate, the cultures were concentrated via centrifugation to facilitate the recovery of resistant mutants. Aliquots were diluted in Tween solution, disaggregated by vortexing for 60 seconds to obtain maximum dispersion and plated onto LB selection plates containing antibiotics to recover resistant bacteria. Amphotericin-resistant *C. albicans* cells were selected on YPD (20 µg/ml Amphotericin B) plates. Colony counts were performed after 48 h of incubation at 37 °C. Mutation frequencies were expressed as the number of antibiotic-resistant mutants recovered as a fraction of the viable count.

DNA sequence analysis of Rif^R mutants.

A single colony from each Rif^R mutants (Supplementary Table S4) was suspended in 50 µL H_2O and the cells were lysed by boiling for 10 min. To identify responsible Rif^R mutations, the region of *rpoB* (β-subunit of RNA polymerase) corresponding to Rif clusters I, II, and III was amplified by PCR using primers PAO1rpoB1 (5′-TCATCGATGTGCTCAAGACC-3′) (Jatsenko *et al*., 2010) and PAO1rpoB3 (5′-GACCGAGTCGATCACACC-3′) (Weigand & Sundin, 2012). PCR products were purified using the QIAquick PCR Purification kit. The primers PAOrpoB1 and PAO1rpoB3 were employed for the sequencing of PCR products.

Mouse model of P. aeruginosa-C. albicans co-infection

Nu/Nu mice (20–22 gr) were purchased from the vivarium of the Institute of Biotechnology, University of México. The mice were housed in filtered cages under specific pathogen conditions and permitted unlimited access to food and water. Prior to animal experiments, *P. aeruginosa PAO1 and C. albicans* CAI-4 were grown for 14 hours to the exponential phase. Next, the cells were pelleted via centrifugation, washed twice with sterile PBS, and the optical density of the cell suspension was adjusted based on spectrophotometry at 600 nm. Mice were inoculated intraperitoneally with each desired dose, which was suspended in 0.2 ml of PBS buffer and mixed immediately before injection. After infection, mortality was monitored over 2 weeks.

8.4 Variantes de *P. aeruginosa* multirresistentes sobreexpresan el transportador multidroga OprM.

Nosotros mostramos que en los biofilms mixtos hay un incremento significativo en la frecuencia de mutantes espontaneas de *P. aeruginosa* resistentes a Tetraciclina (Tc^R) y Cloramfenicol (Cm^R), lo cual correlaciona con un incremento en la mutabilidad de la bacteria (Trejo-Hernández *et al*., 2014). Una mutante Tc^R y una Cm^R fueron aisladas de cocultivos de 24 h para realizar un análisis proteómico con el objetivo de conocer las adaptaciones que le confieren la capacidad de resistencia a antibióticos. La cepa silvestre y las mutantes fueron cultivadas por separado en medio liquido LB a 200 rpm durante 14 h a 37 C. Se realizo extracción fenólica de proteínas celulares totales para ser analizadas mediante geles de dos dimensiones como se describe en Trejo-Hernández *et al*., 2014 (ver material y métodos suplementario). Para identificar las proteínas diferencialmente expresadas por las células Tc^R y Cm^R, la expresión proteica de las células mutantes fueron comparados con la expresión proteica de la cepa silvestre (Tabla 1).

Interesantemente, encontramos que ambas mutantes sobrexpresan la proteína de membrana externa OprM (Figura 7 y Tabla 1). Esta proteína esta codificada en el operon *mexAB-oprM* (Poole *et al*., 1993; Gotoh *et al*., 1995). Las tres bombas expresadas por este operon participan en la extrusión de diferentes antibióticos, incluyendo tetraciclina, cloranfenicol, quinolonas, novobiocin, macrolidos, trimetropim, y β-lactamicos (Poole *et al*., 1993; Gotoh *et al*., 1995; Li *et al*., 1995; Köhler *et al*., 1996; Srikumar *et al*., 1997). La expresión del operon mexAB-oprM es regulada negativamente por la proteína mexR (Poole *et al*.,1996). Ha sido previamente demostrado que la mutación de mexR activa la expresión del operon *mexAB–oprM* y confiere a las células un incremento en la resistencia a diferentes antibióticos (Ziha-Zarifi *et al*., 1999).

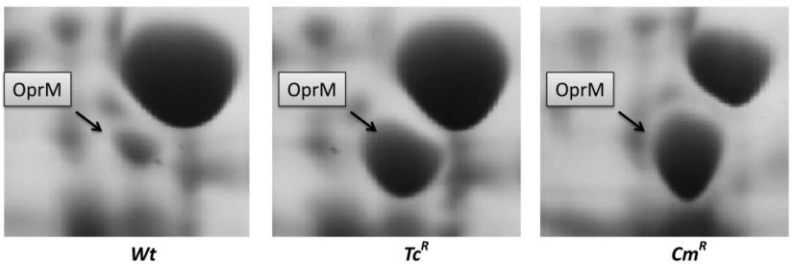


Figura 7. Sobrexpresion de OprM en aislados de *P. aeruginosa* resistentes a tetraciclina (Tc^R) y cloranfenicol (Cm^R) con respecto a la cepa silvestre (Wt).

La adquisición de mutaciones adaptativas es esencial para la persistencia de los microbios durante las infecciones crónicas. Esto es particularmente evidente durante las infecciones por *P. aeruginosa* en los pulmones de pacientes con fibrosis cística (FC) (Ciofu *et al*., 2010). La aparición de cepas resistentes a antibióticos se le atribuye a la mutagénesis causada por especies reactivas de oxigeno generadas por polimorfonucleocitos y por el tratamiento con antibióticos (Mathee *et al*., 1999; Sanders *et al*., 2006; Ciofu *et al*., 2010). Sin embargo, nuestros análisis de mutabilidad en los biofilms mixtos reveló que la competencia interespecífica, es un proceso que favorece la aparición de cepas multirresistentes. Estas mutantes potencialmente pueden ser seleccionadas durante la terapia con antibióticos y cambiar el curso de las infecciones crónicas.

Tabla 1. Proteínas diferencialmente expresadas en mutantes espontaneas resistentes a Tetraciclina (Tc^R) y Cloramfenicol (Cm^R) con respecto a la cepa silvestre.

ORF	Proteína	Descripción	Score	MS sequence coverage %	Fold change
PA0427	OprM	Transportador multidrogas	114	30	8.5
PA4848	AccC	Biotina carboxilasa	121	38	1.75
PA3639	AccA	Acetil-CoA carboxilasa carboxil transferasa	97	47	2.1
PA2968	FabD	Malonil CoA-ACP transacilasa	92	39	1.62
PA0331	IlvA1	Treonina dehidratasa	179	47	1.54
PA0316	SerA	D3-fosfogliceratodeshidrogenasa	121	25	1.73
PA1337	AnsB	Glutaminasa-Asparaginasa	93	23	1.85
PA1584	SdhB	Subunidad β de la succinatodeshidrogenasa	85	41	1.69

8.5 La hiperproducción de piocianina es un rasgo que favorece la adaptación de *P. aeruginosa* en los biofilms mixtos.

Los microbios están sujetos a presiones de selección en el ambiente del hospedero durante el curso de las infecciones crónicas (Giannakis *et al*., 2008; Smith *et al*., 2006). Las características seleccionadas pueden tener un impacto en el desarrollo de la enfermedad, particularmente si ellas confieren un incremento en el bienestar microbiano o en la resistencia a la terapia. Un ejemplo de estos fenómenos es la adaptación de *P. aeruginosa* dentro de las vías respiratorias de personas con FC. Diversos cambios fenotípicos han sido observados entre cepas aisladas de pacientes con

FC, incluyendo cambios en antígenos de superficie (Mahenthiralingam *et al*., 1994), alteraciones en la susceptibilidad a antibióticos (Burns *et al*., 1999), y la sobreproducción de alginato (Govan & Deretic, 1996). Estos cambios adaptativos han sido asociados a las presiones que impone el sistema inmune del hospedero y a las características fisicoquímicas del ambiente en el que se desarrolla la infección. Sin embargo, se desconoce como las interacción interespecífica en las infecciones puede influir en la aparición de características adaptativas que son asociadas con un pronóstico clínico desfavorable. En este trabajo evaluamos la radiación adaptativa de *P. aeruginosa* en los biofilms mixtos. El tamaño, morfología y producción de pigmentos de ~10 000 colonias cultivadas en LB solido (1.5% agar) fueron analizados.

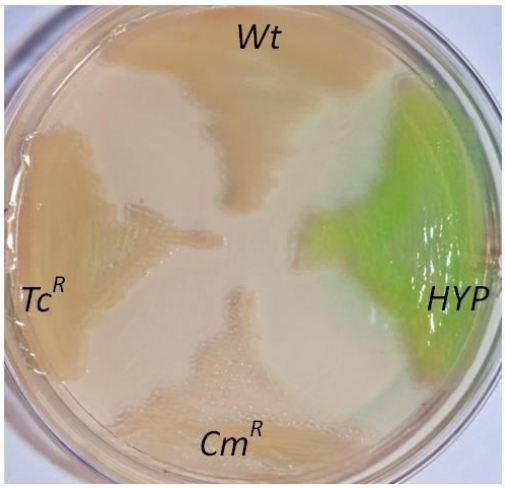


Figura 8. Producción de piocianina por cepas de *P. aeruginosa* aisladas de biofilms mixtos. Cepa silvestre (*wt*), mutante resistente a tectraciclina (Tc^R), mutante resistente a cloranfenicol (Cm^R), mutante hiperproductora de piocianina (HYP). Las cepas fueron cultivadas en LB solido durante 3 días a 37 C.

Observamos un incremento en la abundancia de células que forman colonias traslucidas e hiperpigmentadas (Fig. 8) en los biofilms mixtos de 4 días de cultivo (Fig. 9A). Estas variantes fueron denominadas HYP. Una mutante HYP fue aislada y su patrón de expresión proteica fue analizado y comparado con el de la cepa silvestre (Tabla 2). Encontramos una reducida expresión en la quitinaza ChiC, la proteína de unión a quitina CbpD y las proteínas hipotéticas PA1658 y PA4217 (Fig. 10; Tabla 2). Ha sido reportado que estas proteínas son reguladas por el sistema LasR de *quorum sensing* (Hassett *et al*., 1999). Además, encontramos una disminución

significativa de la expresión de la catalasa CatA, la cual es regulada positivamente por *quorum sensing* (Hassett *et al*., 1999).

Tabla 2. Proteínas diferencialmente expresadas en mutantes hiperproductoras de piocianina (HYP) con respecto a la cepa silvestre.

ORF	Proteína	Descripción	Score	MS sequence coverage %	Fold change
PA2300	ChiC	Quitinasa	123	42	0.42
PA0427	OprM	Transportador multidrogas	113	40	0.18
PA2069	PA2069	Probable carbamoil transferasa	170	32	0.075
PA4236	KatA	Catalasa A	201	56	0.32
PA4236	KatA	Catalasa A	189	50	0.11
PA2444	GlyA2	Hidroximetiltransferasa de serina	87	24	0.41
PA3570	MmsA	Semialdehido deshidrogenasa de metilmalonato	91	30	0.45
PA3735	ThrC	Sintasa de treonina	157	43	0.39
PA0316	SerA	Fosfoglicerato deshidrogenasa	140	40	0.42
PA2001	AtoB	Acetil-CoA acetiltransferasa	122	42	0.37
PA2442	GcvT2	Aminometiltransferasa	137	45	0.48
PA4352	PA4352	Proteina hipotética	125	45	0.41
PA0852	CbpD	Proteina de union a quitina	79	18	0.13
PA2553	PA2553	Probable Acil-CoA tiolasa	135	44	0.23
PA5173	ArcC	Crabamato quinasa	103	38	0.26
PA0865	HpD	4-hidroxifenilpiruvato dioxigenasa	112	27	0.35
PA3326	ClpP2	Proteasa	107	52	0.17
PA0423	PasP	Exoproteasa	90	48	0.19
PA3309	PA3309	Proteina hipotética	116	51	0.44

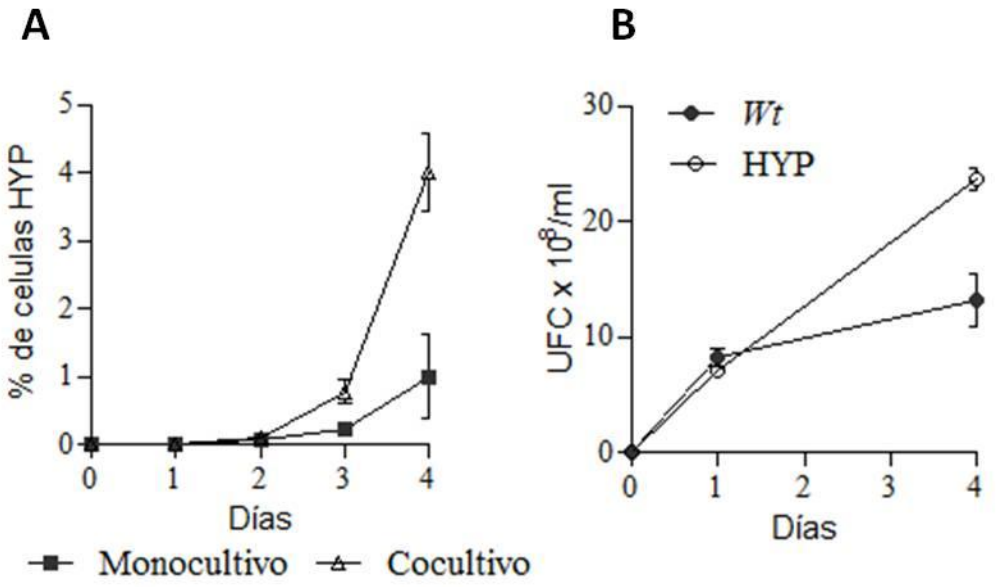


Figura 9. Variantes hiperproductoras de piocianina tienen una ventaja adaptativa en los biofilms mixtos. **A**) Porcentaje de células HYP en cocultivo y monocultivos. **B**) Cambios en el tamaño de población (unidades formadoras de colonias, UFC) de células silvestres y variantes HYP en cocultivos.

En cepas que carecen de un sistema eficiente para detoxificar especies reactivas de oxigeno, Vinckx *et al*., (2009) demostraron que la piocianina juega un papel importante en proteger a las células del estrés oxidativo. De esta manera nosotros proponemos que la hiperproducción de piocianina en mutantes HYP es una respuesta adaptativa para protegerse del estrés oxidativo causado por una significativa disminución de la expresión de la catalasa. Estos resultados son consistentes con trabajos previos en los cuales se ha demostrado que las mutantes LasR desarrollan colonias translucidas e hiperpigmentadas (Cabeen, 2014). En conjunto, nuestros resultados sugieren que las mutantes HYP carecen de un sistema de *quorum sensing* funcional.

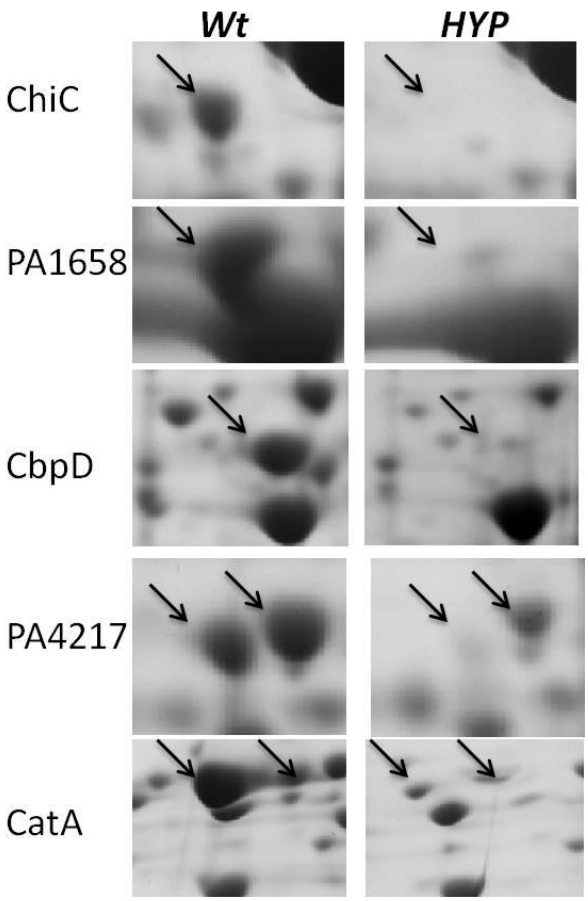


Figura 10. Proteínas diferencialmente expresadas en la cepa HYP. Cada recuadro muestra la expresión de la proteína en la cepa silvestre (wt) y en la cepa hiperproductora de piocianina (HYP).

Aislados de *P. aeruginosa* con mutaciones en el regulador transcripcional LasR son frecuentemente seleccionadas dentro de los pacientes con FC, y las infecciones con estos aislados han sido asociadas con un incremento en la severidad de la enfermedad. Hoffman *et al*. (2010), sugieren que la selección para la perdida de la función de LasR *in vivo*, y el impacto clínico asociado, puede deberse al incremento del crecimiento bacteriano en un ambiente pobre en oxigeno y rico en nitratos. Otros trabajos, han demostrado que la inactivación del *quorum sensing* puede conferir ventajas selectivas al incrementar la viabilidad celular durante la fase estacionaria tardía (Heurlier *et al*., 2005).

Para investigar el valor adaptativo de los rasgos de las variantes HYP, realizamos experimentos en los cuales evaluamos el crecimiento de las cepas HYP en cocultivos de 4 días. La figura 9B

muestra que el tamaño de la población de la variante HYP es significativamente mayor con respecto a la población de la cepa silvestre. Estos resultados sugieren que la inactivación del sistema de *quorum sensing* en *P. aeruginosa* puede ser una estrategia importante para adaptarse a las condiciones ambientales presentes en los biofilms mixtos, y la adquisición de esta adaptación puede verse favorecida por el incremento de la mutabilidad generada por la competencia interespecífica.

Piocianina es uno de los más importantes factores de virulencia de *P. aeruginosa*. Así, el surgimiento y selección de células hiperproductoras de piocianina en los biofilms mixtos, sugiere que la competencia interespecífica es un fenómeno ecológico importante que selecciona rasgos que incrementan la virulencia bacteriana.

9. CONCLUSIONES GENERALES

En el presente trabajo de investigación, demostramos como la competencia por nutrientes, principalmente por hierro, incrementan la producción y expresión de factores que previamente han sido identificados como factores de virulencia en estudios de interacciones hospedero-patógeno. Así, la competencia interespecífica puede ocasionar daños colaterales en el hospedero. Otro rasgo importante observado durante el desarrollo de la interacción, fue el aumento en la frecuencia de mutaciones espontaneas en el hongo y la bacteria en los biofilms mixtos. Mostramos que el incremento de la mutabilidad correlaciona con la represión y la disminución de la actividad de enzimas que protegen al ADN del daño causado por el estrés oxidativo.

Nuestros resultados sugieren que la inactivación del sistema de *quorum sensing* en *P. aeruginosa* puede ser una estrategia importante para adaptarse a las condiciones ambientales presentes en los biofilms mixtos, y la adquisición de esta adaptación puede verse favorecida por el incremento de la mutabilidad generada por la competencia interespecífica.

Así, nuestros resultados sugieren que la competencia entre patógenos oportunistas pueden alterar el curso de las infecciones polimicrobianas incrementando la virulencia, la mutabilidad y la selección de variantes con rasgos que incrementan la virulencia bacteriana.

10. PERSPECTIVAS

Los estudios previos y nuestra investigación nos permitió conocer algunos mecanismos moleculares involucrados en la interacción entre *C. albicans* y *P. aeruginosa*; sin embargo, se desconoce cómo la evolución puede afectar la naturaleza y dinámica de esta interacción y de las interacciones hospedero-microbio-microbio en infecciones polimicrobianas. Por lo tanto resulta evidente la necesidad de conducir análisis evolutivos para complementar los estudios moleculares de las interacciones hongo-bacteria. En conjunto, este conocimiento no solo nos informaría de la biología básica de las interacciones, sino que también podría ser un conocimiento base para el desarrollo de nuevas aplicaciones médicas y biotecnológicas.

Por primera vez, reportamos que *C. albicans* produce una molécula de tipo fenolato, la cual tiene la capacidad de inducir la producción de pioverdina en *P. aeruginosa*. Determinar la estructura molecular de este compuesto nos ayudará a entender los mecanismos por los cuales esta molécula induce la producción del sideroforo pioverdina en *P. aeruginosa*.

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