Departamento de Biología Molecular
Facultad de Ciencias
Universidad Autónoma de Madrid

Sobre el papel del sistema SOS, la recombinación y el intercambio horizontal de genes en la evolución bacteriana

Tesis Doctoral

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Madrid · 2015

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Tesis Doctoral

Presentada por D. Jerónimo Rodríguez Beltrán para optar al grado de Doctor en Ciencias por la Universidad Autónoma de Madrid.

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Realizada en el Departamento de Biotecnología Microbiana del Centro Nacional de Biotecnología del Consejo Superior de Investigaciones Científicas (CNB-CSIC).

“The incredible diversity of life on this planet,
most of which is microbial, can only be understood
in an evolutionary framework”

Carl Woese

Agradecimientos

Gran parte del trabajo aquí presentado ha sido financiado gracias a los proyectos "*Optobacteria: Multianalite automatic system for the detection of drug resistant bacteria*" FP7 286998 y (2014) 968889 y REIPI RD12/0015/0012, concedidos al director de esta tesis, el Prof. Jesús Blázquez, al que debo agradecer que diera su apoyo a un joven estudiante despeinado que llegó al laboratorio buscando un ambiente *friki* donde poder hacer "cosas" con bacterias para su proyecto de fin de carrera. Aquel día fue el principio de una provechosa relación en la que siempre he contado con su confianza, motivo por el que estoy muy agradecido.

En el laboratorio he conocido a gente que me ha ayudado a crecer, tanto científica como personalmente. Particularmente Alexandro y Alex supieron contagiarme de la pasión necesaria para desarrollarme como científico y compartieron conmigo sus personales filosofías de la ciencia. Ellos y todos los compañeros que he tenido a lo largo de estos años, especialmente Coloma, Noe, Ynés, Thuy, Jazz, Elva, Mar, Anabel, Guille y Ani, han tenido la paciencia de trabajar junto a mis excentricidades y han sabido generar el buen ambiente de camaradería tan necesario para compartir los (muchos) fracasos y los (escasos) éxitos que conlleva esta profesión. Coloma se merece mención doble, porque juntos hemos sacado adelante la mayoría de los experimentos de esta tesis (hemos usado tantas placas de Petri, que si apilásemos en una torre sólo las que utilizamos para el tercer artículo, sería seis metros más alta que la puerta de Alcalá).

Fuera del laboratorio, quiero destacar la colaboración a esta tesis de mi Madre, por su apoyo y su insistencia porque la acabara, de los colegas que han aguantado charlas intempestivas sobre biología y la de Djordje y Pin por ser compañeros de carrera, tesis y vida.

Por último, quisiera agradecer a Laura el saber aguantar mi falta de tiempo, que no desconectara en vacaciones y las separaciones forzosas con su sonrisa habitual.

Resumen

El principal objetivo de esta tesis ha sido cuantificar y ampliar el conocimiento sobre los diferentes sistemas evolutivos implicados en el control de la tasa adaptativa en los seres vivos, usando para ello la bacteria modelo *E. coli*. La mutación, la recombinación y la transferencia horizontal de genes son los mecanismos más prominentes de la evolución bacteriana y todos ellos han sido abordados desde diferentes aproximaciones.

En la primera parte de la tesis, se valoró el efecto en el incremento de la mutagénesis que producen ciertos tratamientos antibióticos y si este aumento es dependiente de la presencia de RecA, el activador del sistema SOS. Los resultados sugieren que, al menos ocho antibióticos de los trece ensayados, resultan mutagénicos, en la mayoría de los casos por un mecanismo RecA dependiente.

Después se presentan los resultados relacionados con la caracterización funcional de *dinF*, un gen SOS que probablemente codifica una bomba de expulsión de agentes tóxicos. Se demuestró que su sobreexpresión es capaz de mitigar el estrés oxidativo celular, proteger frente a sales biliares e incluso reducir la tasa de mutación en un mutante *mutT*.

En la tercera sección, se cuantificó la frecuencia de recombinación en una colección de cepas naturales, mediante el uso de una herramienta plasmídica construida *ad hoc*. Los resultados sugieren que la tasa de recombinación es muy diversa y dinámica en *E. coli* y que las cepas patógenas son más propensas a la recombinación que sus congéneres comensales.

En la última parte, se describe un mecanismo de transferencia horizontal de genes mediado por sepiolita, un material arcilloso presente en el pienso animal. Se discuten, además, las potenciales implicaciones con respecto a la diseminación de resistencia a antibióticos y genes de virulencia en el ganado.

Summary

The main objective of this dissertation was to quantify and enlarge the knowledge about the evolutionary systems that control the adaptive rate in all living beings, using the model bacterium *E. coli*. In this work, we have tackled in different ways the most recognized mechanisms of bacterial evolution, namely mutation, recombination and horizontal gene transfer,

In the first part of the dissertation, the increase on mutagenesis that certain antibiotic treatments produce was quantified, assessing whether this increase is dependent on RecA (the SOS response activator). Our results demonstrate that at least eight out of the thirteen antibiotics tested are mutagenic, the most part by a RecA dependent mechanism.

Subsequently, the results concerning the characterization of *dinF,* an SOS gene that probably codes for a toxic compound efflux pump, are presented. We have demonstrated that its over-expression reduces the intracellular oxidative stress levels, protects against bile salts and is able to reduce the mutation rate of a *mutT* deficient strain.

In the third part, the extent of recombination in natural populations of *E. coli*, using a plasmidic genetic tool built *ad hoc* was assessed. Our results suggest that recombination can be very diverse and dynamic in the species. Furthermore, pathogenic strains show greater recombinational potential than their commensal counterparts, suggesting a link between virulence and recombination.

In the last section, a new horizontal gene transfer mechanism mediated by Sepiolite, a clay material commonly used in animal feed is described. We discuss the potential implications that this transfer could represent in terms of the spread of antibiotic resistance and virulence genes.

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Capítulo I
Abreviaturas

Abreviaturas[1]

-Chi o **χ**: Secuencia promotora de entrecruzamientos.

-DNA: Ácido desoxirribonucleico.

-DNAss: ADN de cadena sencilla.

-DSB: Rotura de doble hebra.

-ExPEC: Patógenos extra-intestinales de *E. coli.*

-HGT: Transferencia horizontal de genes.

-IPEC: Patógenos intestinales de *E. coli.*

-MATE: Bombas de expulsión de múltiples drogas y componentes tóxicos.

-MLST: Tipificación multilocus de secuencias.

-**MMR**: Sistema de reparación post-replicativa de emparejamientos erróneos.

-NER: Sistema de reparación por escisión de nucleótidos.

-**RNA**: Ácido ribonucleico.

-ROS: Especies reactivas del oxígeno.

-SSB: Proteína de unión al ADN de cadena sencilla.

1 En el **anexo II** se puede encontrar una disquisición sobre los motivos de la elección de las abreviaturas que aquí se presentan

"Nos esse quasi nanos, gigantium humeris incidentes"[2]

Atribuida a **Bernard de Chartres**

Capítulo II
Introducción

2. Podría ser traducido cómo "somos como enanos a hombros de gigantes", concepto del que más tarde se haría eco Sir Isaac Newton en su archifamosa frase: "If I have seen further it is by standing on the shoulders of giants"

1. Consideraciones iniciales

Vivimos en un mundo dominado por microorganismos que solo existe tal y como lo conocemos gracias a bacterias y arqueas pretéritas que modificaron el ambiente hostil de la Tierra, creando las condiciones que permitieron la aparición y expansión de las formas de vida eucarióticas de las que descendemos. La fuerza directriz de este proceso, así como de todos los que han causado la actual diversidad del planeta, ha sido la evolución. Ésta, de acuerdo con la teoría neodarwinista o síntesis moderna, consiste en la generación espontánea de variantes genéticas y la acción de la selección natural sobre ellas.

Esta tesis versa, a grandes rasgos, sobre los mecanismos responsables de generación de variabilidad en bacterias y su estrecha relación con los sistemas de reparación del DNA. Para ello se ha utilizado como organismo modelo la bacteria *Escherichia coli*, cuyas características generales se exponen en el siguiente apartado.

1.1. *Escherichia coli*

El tracto intestinal humano representa un ambiente propicio para los más de 100 billones de microbios que lo habitan. La mayoría residen en las capas mucosas del intestino grueso, donde se alcanza la mayor densidad poblacional registrada en cualquier ambiente microbiano (10^{11}– 10^{12} células por gramo) (1). El organismo aerobio mayoritario de este hábitat es *Escherichia coli* (2), una bacteria gram negativa, de la familia *Enterobacteriaceae.*

La relación de *E. coli* con su portador es normalmente de comensalismo (3), salvo en individuos inmunocomprometidos o en caso de lesiones que permitan el acceso a otros tejidos (4). Sin embargo, la adquisición de factores de virulencia permiten a *E. coli* convertirse en un organismo patógeno, capaz de causar una gran variedad de infecciones en individuos sanos, entre las que se incluyen diarrea, disentería, septicemia, meningitis, pielonefritis y cistitis (4). Las cepas patógenas de *E. coli* –causantes de más de dos millones de muertes al año (3)– pueden clasificarse en patógenos intestinales (IPEC, por

sus siglas en inglés *intestinal pathogenic E. coli*) y extra-intestinales (ExPEC, del inglés *extraintestinal pathogenic E. coli*).

La gran variedad de nichos que coloniza, así como las diferentes relaciones que adopta con su hospedador, hacen de *E. coli* un buen candidato para estudiar los procesos evolutivos que han generado la gran diversidad y plasticidad de la especie, así como los procesos directrices de la transición del comensalismo a la patogenicidad.

2. Mutación

Obviamente, los mecanismos evolutivos no son únicos en *E. coli,* sino que, con ligeras variaciones, son más bien comunes a todos los seres vivos. De entre ellos destaca la mutación, pues es la causa primera de variabilidad genética. El término mutación fue acuñado por el botánico Hugo de Vries para referirse a los cambios fenotípicos repentinos que observaba en el lapso de una generación en sus trabajos con la planta *Oenothera lamarckiana* (5). Todavía sin conocer la naturaleza física del material hereditario, Max Delbrück conjeturó que las mutaciones serían cambios en el estado cuántico de la "colección de átomos" que conformarían el gen (6). Tras décadas de intensa investigación sabemos que esa colección de átomos es el DNA (7) y que, efectivamente, las mutaciones son cambios fisicoquímicos en su estructura capaces de alterar la información genética.

2.1. Causas y consecuencias moleculares

Molecularmente, el origen de las mutaciones puede ser tanto endógeno como exógeno, siendo el último minoritario salvo en casos de exposición excepcional a agentes genotóxicos (8). Las mutaciones de carácter endógeno, a su vez, pueden deberse a errores nativos de la maquinaria replicativa o a alteraciones bioquímicas. Ambos eventos son

consecuencia de las propiedades fisicoquímicas de la molécula de DNA; en el primer caso debido a su flexibilidad estructural y en el segundo a la gran reactividad que presenta en condiciones fisiológicas de temperatura y pH.

Los errores de la replicación se deben al hecho de que los nucleótidos pueden aparearse entre sí de modo no canónico, a expensas de introducir una distorsión en la molécula de DNA. La frecuencia de aparición de este tipo de eventos es del orden de 10^{-4}–10^{-5} por base y por replicación, según estimaciones *in vitro* (9). Sin embargo, tanto *E. coli* como la mayoría de seres vivos han desarrollado estrategias para lidiar con el alto número de mutaciones potenciales inherentes a la replicación. La acción conjunta de la subunidad correctora del replisoma y del sistema de reparación post-replicativa de emparejamientos erróneos (MMR, del inglés *mismatch repair*) reduce la frecuencia de mutación espontánea *in vivo* prácticamente cinco órdenes de magnitud (10).

En cambio, las alteraciones bioquímicas se deben a que la molécula de DNA presenta numerosas dianas susceptibles de reaccionar con el agua, el oxígeno y, en menor medida con otras moléculas. Por ejemplo, la oxidación de las bases nitrogenadas puede generar decenas de alteraciones. Entre ellas destaca, por su abundancia, la oxidación de la guanina a 8-oxo-guanina, capaz de emparejarse tanto con la citosina como la adenina (11). Como producto de esta ambigüedad se pueden generar transversiones G:C→T:A o A:T→G:C si la 8-oxo-guanina no se elimina a tiempo (12). De nuevo, los seres vivos poseen respuestas especializadas para lidiar con estas lesiones tan comunes; en el caso de *E. coli* el sistema GO es el encargado de detectar y eliminar la 8-oxo-guanina y sus derivados antes o después de haberse incorporado en el DNA: MutT elimina el precursor de nucleótido 8-oxo-dGTP, previniendo su incorporación en el DNA. Por su parte, las glicosilasas MutY y MutM escinden los residuos de 8-oxo-guanina que pudieran haberse incorporado o generado en el DNA naciente (12).

2.2. Hipermutación y evolución

2.2.1. Mutadores estables

La regulación de la tasa de mutación es un parámetro que ha sido de interés por parte de los biólogos evolutivos durante más de ochenta años (13). Como todo parámetro biológico, está sujeto a la acción de la selección natural y, teniendo en cuenta que las mutaciones deletéreas son mucho más probables que las beneficiosas, se ha argumentado que la tasa de mutación debería tender a un nivel óptimo relativamente bajo (14). No obstante, con frecuencia se encuentran aislados naturales hipermutadores que desafían esta teoría (15, 16). La caracterización molecular revela que estas cepas portan defectos en la maquinaria de reparación del DNA [En *E. coli*, principalmente en el MMR (17)] que confieren un marcado aumento de la tasa de mutación. A pesar del tremendo coste de las mutaciones deletéreas, la hipermutación puede ser una estrategia ventajosa en la adaptación a un nuevo nicho o durante la terapia con antibióticos, sobre todo en ausencia de transferencia horizontal de genes (18–20). Tanto es así que la prevalencia de mutadores varía desde un nada desdeñable 1–12% en *E. coli* (15, 16, 21) hasta un 30-50% en aislados de *Pseudomonas aeruginosa* de pacientes con una infección crónica como la fibrosis quística (22, 23). La explicación de este fenómeno radica en la denominada *selección de segundo orden*, que se basa en que la ventaja que confiere un alelo beneficioso hará que aumente su frecuencia en la población, arrastrando consigo al resto de alelos del fondo genético donde se generó. Los mutadores tienen mayor probabilidad de encontrar mutaciones beneficiosas, por lo que su proporción aumentará ligada a la ventaja adaptativa de dichas mutaciones, siempre y cuándo su efecto sea superior al coste de ser mutador (24).

A la luz de los resultados obtenidos en esta tesis, resulta conveniente señalar que existe una marcada diversidad en la frecuencia de mutantes, medida por la capacidad de

generar mutantes espontáneos resistentes a rifampicina, en poblaciones naturales de *E. coli*, aunque no se han encontrado diferencias significativas entre cepas patógenas y comensales (21, 16).

2.2.2. Hipermutación transitoria

Otro tipo de hipermutación, en este caso temporal, es la que sucede cuando se induce el sistema SOS y consecuentemente las polimerasas propensas a error. Aunque se puede encontrar una descripción más detallada de los mecanismos en el capítulo correspondiente al sistema SOS, se tratarán aquí las consecuencias evolutivas de dicho proceso. En resumen, las polimerasas propensas a error son capaces de atravesar lesiones que modifican la disposición estructural del DNA (por ejemplo, dímeros de timina) y bloquean la polimerasa III, permitiendo la supervivencia de la bacteria. Sin embargo, como contrapartida, presentan una baja fidelidad (8). Por qué se han seleccionado variantes con una propensión al error tan alta es un tema aún en debate. Una de las posibles explicaciones es que la baja fidelidad es un corolario inevitable de la habilidad de estas polimerasas para atravesar una gran variedad de lesiones. Dicho de otro modo, la versatilidad a la hora de traspasar daños en el DNA tiene como consecuencia inherente la introducción de mutaciones (25).

Una segunda explicación, consiste en que durante periodos de estrés frecuentes (o suficientemente largos) estos sistemas de mutagénesis transitoria pueden aumentar, a nivel poblacional, la probabilidad de encontrar mutaciones beneficiosas de un modo tan eficiente como los mutadores constitutivos (26). Sin embargo, una vez finalizado el periodo de estrés, el retorno a la replicación no mutagénica evita la acumulación de mutaciones deletéreas y el alto coste asociado que sí deben pagar las bacterias hipermutadoras (20). Por tanto, cabe la posibilidad de que este sistema de reparación mutagénica haya evolucionado para permitir la supervivencia de la bacteria al tiempo que puede ofrecer soluciones adaptativas a nivel poblacional frente a periodos de estrés.

3. Transferencia horizontal de genes

La transferencia horizontal de genes (HGT, del inglés *horizontal gene transfer*) ha sido definida como la transmisión no genealógica de información genética de un individuo a otro (27) y contribuye a la creación de diversidad génica, al permitir a los organismos adquirir genes adicionales a aquellos que heredaron (28). Al contrario que la transferencia vertical (ancestro-descendiente), la HGT no respeta fronteras taxonómicas, pues se han registrado intercambios entre miembros del mismo género (29) e incluso entre diferentes dominios (30). Por tanto, no es sorprendente que el análisis genómico comparativo demuestre que la adquisición de material genético a través de HGT ha sido una fuerza evolutiva predominante en la evolución. En un estudio con 88 genomas procarióticos se estimó que el porcentaje de genes involucrados en HGT puede llegar hasta el 22% (31). Este resultado demuestra que la contribución de la HGT es significativa en la evolución y que, junto con la mutación y los reordenamientos génicos, han dado forma a la actual diversidad bacteriana. Cabe resaltar además el papel clave que ha tenido y tiene la HGT en la diseminación de determinantes de resistencia antibiótica (32, 33) y de virulencia en diferentes linajes bacterianos (34).

3.1. Mecanismos de HGT

Mecanísticamente, se puede catalogar la HGT en tres categorías que serán objeto de los siguientes apartados.

3.1.1. Conjugación

La conjugación bacteriana es la transferencia directa de material genético a través de contacto directo entre dos bacterias. El proceso comienza cuando una célula donadora sintetiza un aparato especializado denominado *pilus conjugativo,* que contacta con la célula receptora y se retrae, atrayendo ambas a un contacto estrecho. En ese punto se establece un puente o poro entre ambas células por el que pasa el DNA a transferir. Éste ha sido previamente cortado de manera específica por lo que se transfiere en forma

monocatenaria (35). Sin embargo, el sustrato de la transferencia no es DNA de cadena sencilla (DNAss, del inglés *single strand DNA*) desnudo, sino unido a proteínas que también son transferidas. Por ejemplo, en su extremo 5' proteínas especializadas denominadas *relaxasas* guían su re-circulación una vez en el interior de la cepa receptora, donde se replicará a doble cadena. Adicionalmente, pueden transferirse proteínas con afinidad por el DNAss como SSB (del inglés *single strand binding protein*) o RecA (36).

3.1.2. Transformación

La transformación natural bacteriana implica la internalización activa de DNAss a partir de DNA de doble cadena extracelular, utilizando para ello un aparato multiproteico especializado (37). La mayoría de las especies que poseen esta habilidad, denominada competencia, lo hacen de forma transitoria y regulada (38).

Aunque *E. coli* se ha considerado tradicionalmente como no competente, lo cierto es que parece poseer la maquinaria necesaria (39) e incluso se han reportado ejemplos de transformación en ambientes “naturales” (40–42). En cualquier caso, es indudable que la transformación bacteriana es un mecanismo de transferencia lateral muy significativo ya que al menos el 1% de las especies bacterianas presentan esta capacidad (43).

Dados los resultados que se presentan en esta tesis, cabe resaltar que algunos compuestos arcillosos, debido a sus propiedades naturales, son capaces de transformar muchas especies bacterianas (44). Este fenómeno, que ha sido principalmente descrito como un protocolo de laboratorio (45, 46), puede ocurrir en determinados ambientes naturales (47).

3.1.3. Transducción

Los bacteriófagos son la entidad biológica más abundante de la biosfera (48) e infectan bacterias inyectando su genoma en el citoplasma. Una vez dentro pueden entrar en dos modos de replicación distintos; el ciclo lítico y el lisogénico. En el segundo caso, el fago integra su DNA en el cromosoma receptor dónde se replicará hasta que, bajo

circunstancias específicas, se induzca el ciclo lítico. En ese momento nuevos viriones son sintetizados utilizando la maquinaria celular. Algunos de ellos empaquetan por error DNA bacteriano en vez del genoma viral, generando las partículas transductoras (49).

Aunque la transferencia de DNA cromosómico por transducción generalizada es un evento poco común (del orden de una vez de cada 10^7–10^9 infecciones), el inmenso número de bacterias y fagos presentes en la biosfera hace este proceso extremadamente frecuente. Al contrario de la conjugación, la transducción no requiere contacto entre la célula receptora y donadora, ni siquiera coexistencia temporal. Es más, se ha determinado que los fagos pueden transducir fragmentos cromosómicos entre diferentes ecosistemas (50). La transformación natural tampoco requiere coexistencia espacial ni temporal, pero la transducción ofrece una ventaja adicional: La cápside protege el DNA albergado en su interior, previniendo su degradación. Sin embargo, los bacteriófagos dependen de receptores específicos en la superficie bacteriana que limitan su tropismo a un espectro generalmente pequeño de especies, lo que disminuye las posibilidades de HGT a especies relativamente lejanas.

4. Recombinación Homóloga

La recombinación homóloga es un proceso biológico crucial para la inmensa mayoría de los seres vivos que implica el apareamiento y transferencia de cadenas entre dos moléculas de DNA que comparten regiones homólogas. Desde su descubrimiento –merecedor de un premio Nobel– por Joshua Lederberg y Edward Tatum en 1947 (51), la recombinación homóloga fue asociada con el proceso sexual de conjugación y entendida como un mecanismo evolutivo para generar diversidad mediante reordenaciones génicas y la dispersión de alelos favorables. No obstante, en las últimas décadas se ha descubierto que la recombinación homóloga es un mecanismo que juega un papel importante en el mantenimiento de la estabilidad génica, ya que actúa en la reparación de lesiones

cómo roturas simples o dobles de la cadena de DNA (52).

Por tanto, la recombinación puede entenderse como un proceso celular cuya función resulta un tanto paradójica, pues según en qué condiciones se dé este proceso puede, por un lado, favorecer el aumento de la variabilidad genética incorporando nuevos alelos y por otro, reparar daños en el DNA de manera no mutagénica, manteniendo la integridad y estabilidad cromosómica.

4.1. Mecanismo molecular de la recombinación

Al menos veinticinco proteínas distintas están implicadas en recombinación homóloga en *E. coli* (53). La gran mayoría tienen homólogos funcionales (aunque no necesariamente estructurales) en los tres dominios de la vida. De hecho, se han encontrado proteínas similares a RecA –la enzima clave del proceso– en prácticamente toda forma de vida examinada hasta la fecha (54).

4.1.1. RecA

El gen *recA* se identificó en *E. coli* en 1965 por Alvin Clark y Ann Margulies (55), mientras buscaban mutantes deficientes en recombinación por conjugación. Años más tarde se elucidó la estructura tridimensional de esta proteína de 352 aminoácidos (56) que ha sido considerada el prototipo de su familia. RecA desempeña un papel principal en los pasos centrales de la reacción de *recombinación*, alineando y emparejando dos moléculas de DNA para después promover el intercambio de hebra (57). La importancia de RecA en este proceso es tal, que cuándo se elimina, la recombinación asociada a la conjugación es 100.000 veces menos eficiente (55). Aunque en otros escenarios la dependencia de RecA puede ser sensiblemente menor, de entre 10 y 100 veces (58).

RecA tiene una afinidad moderada por el DNAss y cuando éste es lo suficientemente abundante, polimeriza de un modo cooperativo sobre él, formando un nucleofilamento proteico RecA-DNAss (figura 1A; paso 1). Este filamento, que es la forma activa de RecA, se conoce tradicionalmente como RecA* y cataliza la invasión de

una molécula de DNAds formando un heterodúplex (compuesto por al menos tres cadenas sencillas) para después facilitar la migración de dicho complejo hasta alcanzar una región en el que la homología de secuencia sea lo suficientemente grande como para catalizar el intercambio de hebras (fig.1A; pasos 2 y 3). En *E. coli* se ha determinado que esta región debe ser de, como mínimo, 23 pb (59). Posteriormente proteínas accesorias se encargan de resolver el intermediario de Holliday resultante y de ligar los diferentes fragmentos de la nueva molécula de DNA recombinante (fig.1A; paso 4).

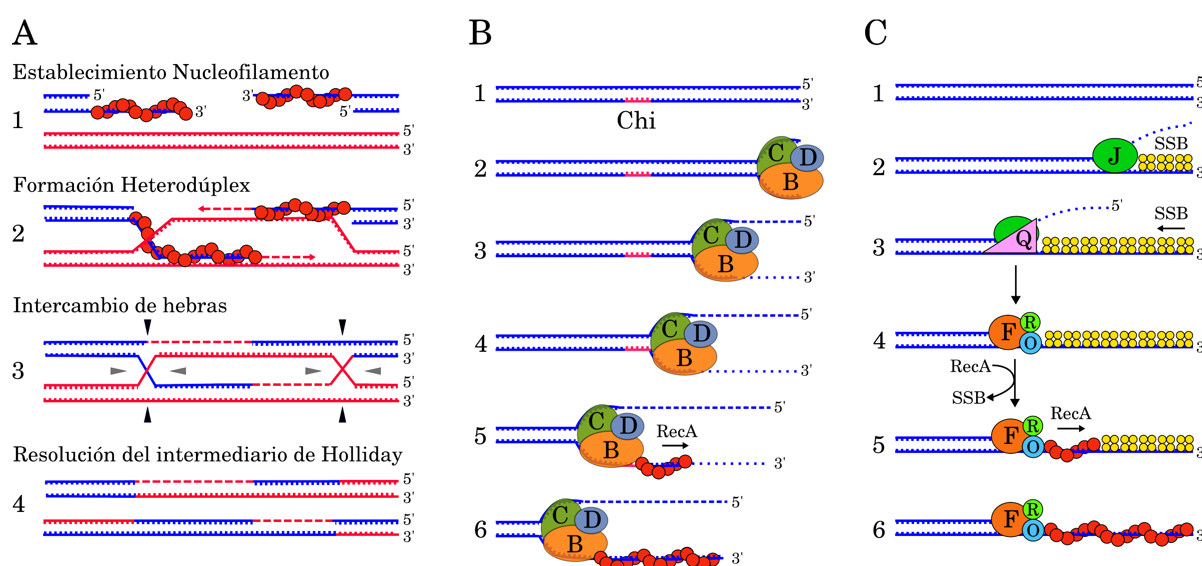

Figura 1. *Esquema general del proceso de recombinación. El modelo representado en A es el de reparación de roturas de doble hebra, pero en su forma general, representa el proceso de recombinación homóloga catalizado por RecA (circulos rojos) en todos los casos. La generación de DNAss puede ocurrir por la vía RecBCD (B) o por la vía RecFOR (C).Ver el texto para una explicación detallada.*

Para que se pueda dar un entrecruzamiento, RecA debe competir por el DNAss con la proteína SSB que presenta mayor afinidad. De hecho, el establecimiento de un complejo SSB-DNAss impide la recombinación *in vitro* (60). Para contrarrestar esta inhibición, existen una serie de proteínas cuya función es la de “cargar” RecA sobre el DNAss reduciendo la barrera cinética impuesta por SSB. En *E. coli*, se han descrito dos vías alternativas de iniciación de la recombinación sobre las que versarán los siguientes apartados.

4.1.2. Ruta RecBCD

La ruta RecBCD es necesaria para más del 99% de los eventos de recombinación asociados a conjugación y transducción, en los que participa DNA bicatenario lineal (54). La deleción de cualquiera de los genes recB o recC inactiva la ruta (61), reduce la recombinación sexual entre 100 y 1.000 veces y aumenta la sensibilidad a agentes que dañan el DNA como la radiación UV y la Mitomicina C (62).

Los principales actores de la ruta RecBCD son la proteína heterotrimérica RecBCD, cuyo sustrato fisiológico es el extremo romo de una molécula de DNA bicatenaria y lineal, es decir una rotura de doble hebra (DSB, del inglés *double strand breaks*) (63), y una secuencia de ocho nucleótidos llamada χ (*Chi*, del inglés *crossover hospot instigator*), que estimula la recombinación (64, 65). RecBCD se une a un extremo de DNA de doble cadena y gracias a su actividad helicasa bipolar separa las hebras mientras se desplaza sobre él (fig.1B; paso 2). Las hebras de cadena sencilla generadas en el paso anterior van siendo degradadas por la subunidad RecB (fig.1B; paso 3) hasta que RecC reconoce la secuencia Chi (5'-GCTGGTGG-3') . Entonces RecC se une fuertemente a la cadena 3', protegiéndola de la degradación por la exonucleasa RecB (fig.1B; paso 4)(66). En el siguiente paso RecBC carga RecA en el DNA de cadena sencilla y comienza el proceso de recombinación (fig.1B; pasos 5 y 6) (67).

4.1.3. Ruta RecFOR

La ruta RecFOR está comparativamente peor caracterizada. No obstante, es esencial para la reparación de roturas de hebra simple o *gaps*. En este caso, parece que la degradación del DNA bicatenario se lleva a cabo por la acción coordinada de la helicasa RecQ y la nucleasa RecJ (fig.1C; pasos 2 y 3), que degradan el DNA produciendo un extremo protuberante 3' (54). Las proteínas encargadas del reclutamiento de RecA forman el complejo RecFOR, que da nombre a la ruta. La proteína RecF reconoce la

unión DNAss-DNAds con un extremo protuberante 3' (68), para después reclutar al complejo RecOR, que al unirse (fig.1C; paso 4), desestabiliza la unión SSB-DNAss permitiendo la nucleación de RecA (69) (fig.1C; paso 5).

Una vez la nucleación ATP-dependiente ha sucedido, la extensión del nucleofilamento ocurre de modo cooperativo y relativamente rápido [2-7 monómeros por segundo (70)] (fig.1C; paso 6) cubriendo todo el DNAss disponible. A partir de este punto, el entrecruzamiento puede comenzar según los pasos anteriormente mencionados.

4.2. Regulación de la Recombinación

La tasa de recombinación, como la de mutación, está fuertemente regulada a nivel celular. Niveles demasiado bajos disminuyen la capacidad celular de reparar el DNA, especialmente en bloqueos de la horquilla replicativa, que son relativamente frecuentes (71). En cambio un exceso puede causar deleciones, duplicaciones, inserciones y translocaciones intracromosómicas que, a la postre, generan inestabilidad genómica y son, en la inmensa mayoría de los casos, deletéreas.

Por ello, la actividad de RecA está regulada en al menos dos niveles diferentes. El primero comprende la regulación transcripcional y será objeto de apartados posteriores. El segundo, mediado por su interacción con otras proteínas y el DNA será considerado en el siguiente apartado.

4.2.1. Autoregulación estructural de RecA

La estructura terciaria de RecA contiene un dominio desordenado en su parte C-terminal. Este dominio actúa como una “solapa” autoreguladora (denominado en inglés *autoregulatory flap*). Cuándo se eliminan los veinticinco aminoácidos que conforman esta región, casi todas las actividades de RecA se ven incrementadas, particularmente la unión a DNA (72) y la capacidad para desplazar a SSB (73) lo que produce un incremento significativo de la tasa de recombinación (74).

Aparte de dicha deleción, se han caracterizado mutantes puntuales en otros dominios capaces de incrementar la frecuencia de recombinación medida en experimentos de conjugación. Por ejemplo, la sustitución D112R, que afecta a un *loop* entre dos hélices alfa, aumenta más de cincuenta veces la tasa de recombinación sin afectar a la inducción SOS (74, 75). Del mismo modo, la expresión heteróloga de RecA de *P. aeruginosa* en *E. coli* aumenta la recombinación más de cuarenta veces (74). Esto sugiere que la actividad de RecA ha llegado a un óptimo distinto en cada especie (y quizás en cada ambiente) que probablemente responde a un compromiso entre reparación y generación de diversidad.

4.2.2. Regulación por otros factores

Aunque los componentes de las rutas RecBCD y RecFOR no son reguladores *sensu stricto*, su interacción dinámica con RecA les convierte en un factor determinante en la bioquímica de las reacciones de entrecruzamiento. El grado de su actividad depende de factores complejos, pero en el caso de la ruta RecBCD cabe destacar la importancia de la secuencia Chi como regulador de la recombinación (76). La secuencia Chi está sobrerepresentada en el genoma de *E. coli* K-12. Se puede encontrar en 1.009 ocasiones en MG1655 (77), un 8.000% más de lo que se esperaría por azar para cualquier secuencia de ocho nucleótidos (78). Teniendo en cuenta que la secuencia Chi regula en gran medida la recombinación mediada por RecBCD, resulta plausible que variaciones en el número de estas secuencias por genoma generen diferencias en la frecuencia de recombinación espontánea de cada cepa

Otro ejemplo es la proteína SSB, que tiene un carácter antagónico pues compite por el DNAss con RecA. Sin embargo, la actividad de SSB no es siempre inhibidora; RecA no es capaz de unirse al DNAss con estructura secundaria fuerte y, al menos *in vitro*, la adición de SSB elimina la estructura secundaria permitiendo la nucleación de RecA (60, 79).

4.3. Importancia Evolutiva

En el presente apartado se presentan las principales hipótesis que justifican la evolución y mantenimiento del *sexo bacteriano*, entendido como la acción conjunta de la transferencia de DNA por los mecanismos descritos en el apartado "Transferencia horizontal de genes" y la recombinación. Mediante este proceso las bacterias son capaces de incorporar en su genoma rasgos completamente nuevos o simplemente nuevos alelos de los genes ya presentes. Sin embargo, el proceso de obtención y asimilación de material génico exógeno conlleva un coste atribuible varios factores.

Por ejemplo, en el escenario más simple, la adquisición de grandes fragmentos génicos conlleva un coste metabólico asociado a la replicación del nuevo DNA, pero sobre todo a su transcripción (80) y traducción (81). Además, debido a la falta de optimización, el nuevo material puede ser deletéreo al presentar un uso de codones distinto (82) o codificar para proteínas que no adopten un plegamiento correcto en el hospedador (83). De un modo más específico, el material transferido puede alterar las redes de interacción entre proteínas (84) o de regulación de la expresión (85), lo que supone un coste relativamente grande. Obviamente, si la expresión de los genes adquiridos es alta, su efecto puede ser incluso más perjudicial, pues se exacerban los factores anteriormente mencionados (86).

Estos datos apuntan a que la recombinación y la HGT son, como la mutación, deletéreos en la mayoría de los casos y a que sólo en contadas ocasiones el material transferido constituye una ventaja adaptativa. Sin embargo, a diferencia de la mutación, que es un proceso inherente a la replicación del DNA y por tanto, inevitable hasta cierto punto, el sexo bacteriano es un proceso activo y debe representar algún beneficio para los organismos involucrados.

Existen varías teorías que justifican por qué la evolución ha desarrollado y mantenido el proceso de sexual y por qué éste puede ser beneficioso para las poblaciones bacterianas. A continuación se exponen de manera somera algunas de ellas.

Una de las teorías más aceptadas para justificar el sexo es el *modelo de Fisher-Muller* (87, 88), en el que se estipula que la recombinación puede reducir la interferencia clonal que se genera cuándo ocurren mutaciones beneficiosas en la misma población pero en distintos linajes, por lo que deben competir entre ellos por alcanzar la fijación. Esta competición ralentiza el incremento global en *fitness* (89, 90). En cambio, en poblaciones sexuales aumenta la probabilidad de que varios alelos beneficiosos coincidan en el mismo linaje, lo que acelerará la adaptación global de la población. Varias aproximaciones teóricas (91, 92) y experimentales (93) apoyan esta hipótesis.

Otra posible explicación radica en el hecho de que poblaciones asexuales inevitablemente acumulan mutaciones deletéreas a una tasa mayor que beneficiosas, lo que disminuye el *fitness* global. Incluso aquellos linajes portadores de alelos beneficiosos irán acumulando mutaciones que lastrarán su éxito evolutivo. Esta hipótesis, que se conoce como *trinquete de Muller* (91) en referencia a la irreversibilidad del proceso y a su principal valedor (94), ha sido demostrada experimentalmente en bacterias (95). En poblaciones sexuales, en cambio, la teoría predice que la relevancia de este fenómeno se ve mermada, puesto que los alelos beneficiosos pueden recombinar en un genoma libre de mutaciones perjudiciales (91).

Los modelos anteriormente mencionados consideran la adaptación a un ecosistema estable. En cambio, parece lógico suponer que el escenario es, en general, más variable, si tenemos en cuenta que la mayoría de especies bacterianas viven en comunidades microbianas complejas donde la selección por especies competidoras, predadores, parásitos o el sistema inmune es fuerte y continua. La evolución en este tipo de ecosistemas sucede, en ocasiones, de un modo similar a una “carrera armamentística”, en el que la adaptación de una especie (p. ej., infectividad) ejerce presión selectiva para la contra-adaptación de otra (p. ej., resistencia). Esta coevolución antagonista se ha usado para explicar el sexo en eucariotas (96) bajo la denominación de *hipótesis de la Reina Roja* y podría ser válido también para procariotas. La presión del sistema inmune puede

llevar a las bacterias a remodelar su superficie, buscando no ser detectadas, lo que empuja al sistema inmune a explotar nuevas dianas. Al contrario que la mutación, que supone en general cambios pequeños, mediante la recombinación se pueden obtener alelos muy diferentes en un sólo evento, lo que acelera esta carrera armamentística (97). En la misma linea argumental, la fuerte selección producida por el tratamiento antibiótico podría seleccionar variantes hiperrecombinadoras, puesto que tienen mayor oportunidad de incorporar genes de resistencia.

Estos tres modelos (y otros no tratados aquí) tratan de explicar la ubicuidad del sexo y no deben considerarse excluyentes entre sí, sino que la explicación más razonable es, probablemente, multifactorial.

5. La respuesta SOS

Hace más de 60 años, Jean Weigle hizo un descubrimiento pionero al observar que la supervivencia del bacteriófago λ inactivado mediante radiación UV aumentaba drásticamente cuando las células huésped también eran irradiadas. Entre los fagos "reactivados" encontró, además, una cantidad elevada de mutaciones, hecho que fue conocido posteriormente como *mutagénesis de Weigle* (98). A finales de la década de los sesenta, Evelyn Witkin sugirió la existencia de un sistema inducido por daño en el DNA responsable de la filamentación de *E. coli* B tras su irradiación con luz UV (99) y se descubrió que la mutagénesis de Weigle era dependiente de *lexA* (100) y de *recA* (101).

Pero fue en 1970 cuando, en una carta privada enviada a algunos colegas (cuyo fácsimil fue finalmente publicado en la ref. 102), Miroslav Radman propuso la existencia de un mecanismo inducible de reparación del DNA controlado por la acción de los genes *lexA* y *recA* que estaría acompañado de mutagénesis para el que acuñó la expresión

"reparación SOS"[2]. Desde aquel momento, los estudios del sistema SOS empezaron a perfilar el modelo actual que se presenta a continuación.

5.1. Señal inductora de la respuesta SOS

La regulación del sistema SOS depende principalmente de la relación de tres moléculas: el DNAss y las proteínas RecA y LexA.

El DNAss es la señal que la bacteria interpreta como señal unívoca de daño en DNA, actuando como "disparador". Este DNAss se genera principalmente como consecuencia de la acción de los complejos presinápticos RecBCD o RecFOR en respuesta a una DSB, o bien un *nick* o rotura de hebra simple, respectivamente.

5.1.1. Inducción del SOS en ausencia de agentes genotóxicos

Conviene resaltar que, aunque el DNAss es el "disparador" del SOS, el daño que genera esta señal puede ser tanto exógeno como endógeno. Así, en ausencia de agentes genotóxicos, aproximadamente el 1% de células en fase exponencial de *E. coli* presentan el regulón SOS inducido (103). De hecho, se ha descrito que hasta el 7.5% de las células sufren roturas espontáneas de doble hebra en condiciones de crecimiento estándar de laboratorio (104). La mayoría de los factores implicados en la inducción del SOS en ausencia de agentes externos están asociados a bloqueos de la horquilla replicativa, producidos generalmente por colisiones con aductos voluminosos. Un ejemplo son las colisiones del replisoma con la RNA polimerasa, que ocurren porque ambas maquinarias se desplazan a distintas velocidades y, en ocasiones, en sentido opuesto (105). Este hecho está aparentemente relacionado con la tendencia de los genes con elevada transcripción a estar alineados con la dirección de la replicación (106). Incluso en células que no están dividiéndose activamente, la formación de híbridos de ARN y DNA durante la transcripción puede acabar generando roturas de doble hebra que acarrean la activación del

2. En la presente tesis se utilizan cómo equivalentes a dicha expresión los términos sistema, regulón o respuesta SOS dado que están actualmente muy generalizados.

SOS (107).

5.1.2. Inducción del SOS mediada por agentes genotóxicos

Los agentes genotóxicos son, quizás, el ejemplo más paradigmático y clásico de inductores del sistema SOS. Se pueden clasificar en dos categorías relativamente extensas: agentes físicos y agentes químicos. La primera categoría engloba radiaciones como los rayos Gamma (108) o los rayos X (109) y la luz UV, que es uno de los agentes físicos mejor estudiados. Además, condiciones ambientales, como la alta presión hidrostática (110), pueden inducir el sistema mediante la activación de nucleasas específicas (111).

Con respecto a los agentes químicos, el daño oxidativo, de origen tanto endógeno como exógeno, es una de las alteraciones más comunes que ha de sufrir el DNA. De hecho se estima que, en condiciones de crecimiento aeróbico, cada célula de *E. coli* tiene que lidiar con ~4000 lesiones de carácter oxidativo por división (112). Los mecanismos para reparar esta ingente cantidad de daño son múltiples e incluso redundantes (113). Así, no toda lesión oxidativa conlleva una inducción del SOS, dado que existe maquinaria especializada para su reparación. En cambio, cuando esta maquinaria falla o se satura, puede dar lugar a DSB al provocar bloqueos de la horquilla de replicación (114). El peróxido de hidrógeno, por ejemplo, es un conocido inductor de la respuesta SOS (115, 116).

Ciertos antibióticos son capaces de inducir la respuesta SOS, en rangos de concentraciones relativamente amplios. Algunos de ellos inducen el sistema porque su modo de acción implica una alteración de la maquinaria replicativa. Por ejemplo, las quinolonas como el ciprofloxacino causan el bloqueo de la replicación al obstruir las topoisomerasas de tipo II, lo que conlleva roturas de doble hebra (117). La trimetoprima o las sulfonamidas inhiben pasos clave de la ruta de síntesis del tetrahidrofolato, un precursor de las bases nitrogenadas sin el cuál la célula es incapaz de sintetizar timina (118). Bajo estas circunstancias la replicación se ve afectada y el SOS inducido (119). Sin embargo, el

mecanismo a través del cual otras clases de antibióticos son capaces de generar la inducción del SOS no es tan directo. Por ejemplo, los β-lactámicos, que inhiben la síntesis de la pared bacteriana, pueden inducir el SOS a través del sistema de dos componentes DpiAB, que es capaz de bloquear la replicación (120). Esta inducción, por tanto, no es directamente dependiente del daño en el DNA. Conviene reseñar que la inhibición de la síntesis de proteínas por antibióticos como los aminoglucósidos y el cloranfenicol puede inducir también la respuesta SOS en *Vibrio cholerae* (121), *Photorhabdus luminescens* y *Klebsiella pneumoniae* (122), pero no en *E. coli* (123).

5.2. Regulación de la respuesta SOS

5.2.1. Regulación transcripcional

En respuesta al daño en el DNA, RecA se une al DNAss formando el filamento nucleoproteico helicoidal RecA-DNAss. Este filamento, además de mediar la recombinación, es capaz de activar la capacidad autoproteolítica de LexA, el represor del sistema SOS (124). La proteólisis da lugar a dos cadenas polipeptídicas sin actividad, lo que libera la expresión del sistema. En condiciones homeostáticas (es decir, en ausencia de daño en el DNA), el represor LexA se mantiene unido en forma homodimérica a una secuencia denominada *caja SOS* situada en la región operadora de los genes del sistema (125). Variaciones en la secuencia consenso (5'-TACTGT$(AT)_4$ACAGTA-3' en *E. coli*) determinan la fuerza con la que LexA es capaz de reprimir cada uno de los genes, y por tanto la abundancia de sus respectivas proteínas en el estado no inducido, además del orden en el que se liberan de la represión (126–128).

5.2.2. Regulación post-transcripcional

El control de la respuesta SOS sucede a varios niveles. Como ya se ha comentado, la interacción de LexA y RecA y el acceso de RecA al DNAss representan un papel preponderante en su regulación. El presente apartado, en cambio, comprende las interacciones con otras proteínas una vez que el filamento nucleoproteico se ha establecido. Por

ejemplo, RecX y DinI regulan de modo opuesto la estabilidad del filamento RecA-DNAss. Tanto el gen *recX* como *dinI* forman parte de regulón SOS (129, 130) y, mientras que RecX actúa bloqueando la extensión del filamento nucleoproteico durante su ensamblaje (131), DinI parece estabilizarlo (132). Además, DinI inhibe el corte de UmuD hacia UmuD' (133) lo que sugiere que podría retrasar la síntesis translesión, ganando tiempo para permitir que la reparación de alta fidelidad tenga lugar (57).

Otras proteínas con efecto inhibitorio sobre la respuesta SOS son RdgC y PsiB. La primera inhibe, al menos *in vitro*, el corte autoproteolítico de LexA (134). El mecanismo de acción de PsiB no está claro, pero está codificada en un gen asociado a plásmidos conjugativos, cuya función podría ser inhibir la inducción tras la transferencia de DNAss durante la conjugación (135, 136).

5.3. Funciones del regulón SOS

La excepcional regulación del sistema SOS (tanto a nivel transcripcional como post-transcripcional) previene falsos disparos y asegura una inducción proporcional al daño en el DNA (137).

El regulón SOS coordina la expresión de al menos 57 genes de los que más del 50% no tienen todavía una función asignada (138). Entre los caracterizados, muchos están relacionados con la reparación o la tolerancia al daño en el DNA (138–140).

Por ejemplo, en los primeros momentos de la inducción SOS se activa la transcripción de genes cuya función es reparar lesiones en el DNA de manera no mutagénica, como el sistema de reparación por escisión de nucleótidos (NER, del inglés, *nucleotide excision repair*), compuesto por los genes *uvrA*, *uvrB* y *uvrD* (8).

Si el daño es persistente, se activan DNA polimerasas especializadas capaces de replicar el DNA incluso cuándo la hebra molde está muy dañada, aunque como contrapartida presentan una baja fidelidad. Estas polimerasas se conocen como *polimerasas propensas a error* y llevan a cabo un proceso comúnmente denominado síntesis trans-

lesión. En *E. coli* hasta la fecha se han descrito tres. La polimerasa II, codificada en el gen polB es la única de las tres que posee actividad correctora de errores 3'-5', aunque aun así es mutagénica en regiones ricas en AT (141).

La polimerasa IV, cuyo gen *dinB* fue de los primeros genes en ser identificado (129), presenta un fenotipo poco marcado en la mutagénesis SOS (142), aunque está fuertemente implicada en la mutación adaptativa mediada por RpoS (143, 144).

La última polimerasa descrita en E. coli está codificada en el operón *umuDC*. Para formar la forma activa de Pol V, UmuD debe sufrir un corte autoproteolítico mediado por RecA* en sus primeros 24 aminoácidos para dar lugar a UmuD' (145). Tras la unión de UmuC, la polimerasa, formada por el heterotrímero $UmuD'_2C$, aún requiere la transferencia de un monómero de RecA unido a ATP para ser procesiva en la síntesis translesión (146). Esta regulación tan compleja responde probablemente a la necesidad celular de limitar la síntesis de DNA mutagénica al mínimo (147).

Adicionalmente, $UmuD_2$ podría forma parte de un punto de control, deteniendo la síntesis de DNA por medio de su interacción con la subunidad beta de la polimerasa III (148). De ser cierto, resulta interesante hacer notar la similitud de este punto de control procariótico con el eucariótico, orquestado por la proteína p21 (149).

Además, mediante un incremento notable de la expresión del gen SOS *sulA* (125) la proteína SulA inhibe la septación mediante su interacción con FtsZ. El resultado es un elongamiento celular conocido como filamentación, que podría actuar sinérgicamente con el control por $UmuD_2$ para inhibir la división bacteriana.

"[...] *one starts at point A and moves toward the goal at point B. Soon enough, things move off course, and the path meanders and loops back. Experiments stop working, all assumptions seem wrong, and nothing makes sense.* [...] *Then, in the midst of confusion, one senses a new problem in the materials at hand. Let's call this new problem C. If C is more interesting and feasible than B, one can choose to go toward it. After a few more detours, C is reached. The researchers can pause to celebrate before taking time to think about the next problem.*"

Uri Alon
How to Choose a Good Scientific Problem

Capítulo III Objetivos

1.- Definir qué antibióticos (o al menos qué familias) son capaces de inducir mutagénesis en *E. coli* y en qué medida después de un tratamiento con concentraciones en torno a la mínima inhibitoria.

2.- Determinar cuál es la contribución de RecA en la resistencia a antimicrobianos, la filamentación y en la mutagénesis que algunos producen. Evaluar el potencial terapéutico que representaría su inhibición.

3.- Caracterizar el fenotipo del gen SOS con función desconocida *dinF*. Comprobar si, como su homólogo *norM*, tiene un papel fisiológico en la detoxificación de especies reactivas del oxígeno.

4.- Diseñar y construir una herramienta plasmídica para medir eventos de recombinación e introducirla en cepas naturales de *E. coli*. Validar su funcionamiento, valorando posibles factores de confusión como el número de copias o la dependencia de RecA.

5.- Analizar cuál es la distribución de frecuencias de recombinación en poblaciones naturales de *E. coli* para después determinar si existen diferencias entre subgrupos de cepas atribuibles a su origen de aislamiento, patotipo, perfil de virulencia o grupo filogenético.

6.- Comprobar si agentes abióticos, como los materiales arcillosos, son capaces de transferir material genético directamente de una célula donadora a una receptora.

7.- Estudiar la plausibilidad en escenarios naturales y las posibles consecuencias de la transferencia mediada por arcillas en el contexto de la diseminación de genes de virulencia y resistencia a antibióticos

“You have to know how to accept rejection and reject acceptance”

Ray Bradbury
Advice to Writers

Capítulo IV
Publicaciones

A continuación se presentan las publicaciones que han dado lugar a esta tesis. En la primera sección se analiza el potencial que presentan las estrategias orientadas a inhibir *recA*, enfocando principalmente la mutagénesis producida por concentraciones de varios antibióticos de uso clínico y, por tanto, el aumento de aparición de bacterias resistentes.

En la segunda sección se presenta la caracterización de *dinF*, un gen del sistema SOS, que está implicado en la protección contra dos de los insultos más comunes a los que ha de enfrentarse una bacteria residente mayoritariamente en el sistema digestivo; el estrés oxidativo y las sales biliares.

La tercera sección de este capítulo se ocupa de caracterizar la magnitud de la recombinación en cepas naturales de *E. coli*. Para ello se construyó un sistema capaz de medir eventos de recombinación y se aplicó a una colección de cepas naturales, tanto de origen patógeno como comensal.

La cuarta y última sección, compuesta por dos artículos, describe el descubrimiento de que la sepiolita –una arcilla presente en el pienso animal– es capaz de mediar la transferencia horizontal de plásmidos entre diferentes especies y se discuten las implicaciones que tiene en la diseminación de factores de virulencia y resistencia antibiótica.

1. Efecto de la inactivación de *recA* sobre la mutagénesis producida por concentraciones subinhibitorias de antimicrobianos en *Escherichia coli*

En el artículo que se presenta a continuación se describen una serie de experimentos enfocados a dilucidar cual es la contribución de RecA a la mutagénesis mediada por concentraciones subinhibitorias de diversos antibióticos. Antes de la realización de este trabajo ya era sobradamente conocida la participación de las polimerasas propensas a error controladas por el SOS en el aumento en mutagénesis producido por ciertos antibióticos (particularmente del grupo de las quinolonas) (150, 151). Además, varios autores habían propuesto la inactivación o inhibición de *recA* como una estrategia terapéutica a desarrollar (152), pues ensayos de infección *in vivo* demuestran que la inducción del SOS contribuye al desarrollo de resistencia antibiótica y al consecuente fracaso clínico (153). Tomando estas dos ideas cómo punto de anclaje, decidimos estudiar qué papel tiene RecA en el aumento de la frecuencia de mutantes inducida por concentraciones en torno a la concentración mínima inhibitoria de un nutrido grupo de antibióticos de uso clínico

Los resultados señalan que muchos de los antibióticos ensayados son capaces de estimular la aparición de mutantes (ocho de trece), aunque en la mayoría de los casos de manera moderada. En cambio, algunos de ellos (particularmente el trimetoprima y el sulfametoxazol) son capaces de generar un aumento en la mutagénesis de hasta 20 veces coincidente con una fuerte activación del sistema SOS. En casi todos los casos, la inactivación de *recA* elimina la mutagénesis mediada por estos antibióticos, reduciendo la tasa de generación de mutantes a niveles similares a los obtenidos para los cultivos no tratados.

Los resultados obtenidos en este trabajo ayudan a esclarecer la participación de RecA y la respuesta SOS en el aumento de la variabilidad genética por mutación en las poblaciones bacterianas sometidas a tratamiento antibiótico. Dado que dicho aumento ha sido relacionado con una mayor probabilidad de fracaso terapéutico, la inhibición de RecA parece un candidato ideal para el desarrollo de nuevos fármacos que podrían usarse como adyuvantes en el tratamiento antibiótico.

"What does not kill me, makes me stronger."

Friedrich Nietzsche

J Antimicrob Chemother 2011; **66**: 531–538
doi:10.1093/jac/dkq496 Advance Access publication 5 January 2011

Journal of
Antimicrobial
Chemotherapy

Effect of *recA* inactivation on mutagenesis of *Escherichia coli* exposed to sublethal concentrations of antimicrobials

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Received 4 August 2010; returned 17 September 2010; revised 30 November 2010; accepted 30 November 2010

Objectives: Low concentrations of some antibiotics have been reported to stimulate mutagenesis and recombination, which may facilitate bacterial adaptation to different types of stress, including antibiotic pressure. However, the mutagenic effect of most of the currently used antibiotics remains untested. Furthermore, it is known that in many bacteria, including *Escherichia coli*, stimulation of mutagenesis is mediated by the SOS response. Thus, blockage or attenuation of this response through the inhibition of RecA has been proposed as a possible therapeutic adjuvant in combined therapy to reduce the ability to generate antibiotic-resistant mutants. The aim of this work was to study the capacity of sublethal concentrations of antimicrobials of different families with different molecular targets to increase the mutant frequency of *E. coli*, and the effect that inactivation of *recA* would have on antibiotic-mediated mutagenesis.

Methods: We tested the mutagenicity of the following antimicrobials: ampicillin; ceftazidime; imipenem; fosfomycin; ciprofloxacin; trimethoprim; sulfamethoxazole; trimethoprim/sulfamethoxazole; colistin; tetracycline; gentamicin; rifampicin; and chloramphenicol.

Results: Eight out of the 13 antimicrobials tested stimulate *E. coli* mutagenesis (slightly in most cases), with trimethoprim, alone or in combination with sulfamethoxazole, producing the highest effect. Inactivation of *recA* abolishes the mutagenic effect and also produces increased susceptibility to some of the tested antimicrobials.

Conclusions: The fact that inactivation of *recA* reduces mutagenicity and/or increases the activity of a large number of antimicrobials supports the hypothesis that RecA inhibition might have favourable effects on antibiotic therapy.

Keywords: mutations, antibiotic resistance, trimethoprim, sulfamethoxazole

Introduction

It has been demonstrated that some antimicrobials, such as fluoroquinolones and β-lactams, increase mutagenesis in bacteria via the induction of error-prone DNA polymerase expression.[1–3] Also, we have shown that the cephalosporin ceftazidime elicits adaptive responses, including increases in mutant frequency, in *Pseudomonas aeruginosa*.[4] In addition, it is known that subinhibitory concentrations of the fluoroquinolone antibiotic ciprofloxacin promote genetic recombination in *Escherichia coli*.[5,6] Very recently, Kohanski *et al*.[3] have demonstrated that sublethal concentrations of some bactericidal antibiotics induce mutagenesis and that this induction correlates with an increase in reactive oxygen species (ROS), which in turn produces induction of the SOS response.[7] Therefore, it is likely that some antibiotic treatments may influence the appearance of antibiotic-resistant bacteria through SOS mutagenesis [8–10] or a combined effect of ROS and SOS mutagenesis.[11] Consequently, abolition or attenuation of the SOS response has been proposed as a possible therapeutic adjuvant in combined therapy to reduce the capacity to generate antibiotic-resistant mutants.[8,12,13]

In many bacterial species, such as *E. coli*, DNA-damaging agents trigger the SOS response, which involves the induction of *recA* expression. Contact with single-stranded DNA (ssDNA) activates the coprotease activity of the RecA protein, which promotes self-cleavage of LexA, the SOS transcriptional repressor, and leads to increased transcription of the SOS response regulon.[14,15] The autogenous control of *lexA* transcription

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supports a cellular response that is exquisitely proportional to the DNA damage level and prevents false triggering of the SOS response.[16] RecA has multiple functions that affect different cellular processes, such as the rescue of stalled replication forks,[17,18] coprotease action involved in the self-cleavage of LexA and UmuD,[19] promotion of homologous recombination,[20] control of swarming motility[21] and the behaviour of bacteria in biofilms.[22]

In this work we explored whether low concentrations of a representative group of antimicrobials of different classes may contribute to genetic variation by affecting mutagenesis in *E. coli* and, as a consequence, favour the emergence of antibiotic resistance by mutation. In addition, we studied whether inactivation of *recA* reduces or prevents this induced mutagenesis. The tested antimicrobials or combinations were ampicillin, ceftazidime, imipenem, fosfomycin, ciprofloxacin, trimethoprim, sulfamethoxazole, trimethoprim/sulfamethoxazole, colistin, tetracycline, gentamicin, rifampicin and chloramphenicol.

Materials and methods

Bacterial strains, plasmids and medium

The *E. coli* K-12 strains used were MG1655, ME12 (MG1655 *lacZΔC-lacZΔN-yfp*) and an ME12 Δ*recA*::*kan* derivative.[23] The construction of ME12 strains has previously been described.[6,23] The *recA* phenotype was verified by measuring UV sensitivity. The plasmid pSC101-P*recA*::GFP harbours a green fluorescent protein (GFP) transcriptional fusion after the promoter of the *recA* gene.[24] Luria-Bertani medium (LB) was prepared according to Miller.[25] MICs of antibiotics for ME12 and ME12*recA* were determined according to CLSI recommendations,[26] except that the bacterial inocula were identical to those used in all subsequent mutagenesis experiments. Antibiotics tested for stimulation of mutation were used at different concentrations around their MICs.

Mutagenesis experiments

Mutant frequencies were studied as described.[6,23] Briefly, for mutant frequency measurement, 2 mL aliquots of exponentially growing cells (10^8 cells/mL) were incubated with different concentrations of antibiotic for 4 h at 37°C with shaking (250 rpm). One mL of these cultures was centrifuged for 10 min at 6000 rpm in a minifuge. The pellet was resuspended in 2 mL of fresh LB medium and incubated overnight at 37°C with shaking. This step is necessary to resolve the filaments formed after treatment with some antibiotics, such as ciprofloxacin, ceftazidime, trimethoprim and trimethoprim/sulfamethoxazole. Resolution of filaments was verified by direct observation of samples from the different cultures under the microscope. Only cultures with a proportion of filaments of <5% of total cells were plated. Viable cells were determined by plating appropriate dilutions onto LB agar plates. Mutant frequencies were calculated as the number of colonies growing on rifampicin (100 mg/L) or fosfomycin (10 mg/L) plates per viable colony. At least three independent experiments were performed for each antibiotic concentration, and three more, with five replicas each, for the most mutagenic concentrations observed. For the experiments with the *recA* mutant, five independent experiments were performed for each concentration.

Effect of antibiotics on recA expression

To qualitatively assess the antibiotic-mediated induction of transcription from the *recA* promoter the strain ME12 containing the pSC101-P*recA*::GFP reporter plasmid was used. A 100 μL aliquot of an overnight culture was inoculated into LB soft agar (0.7% agar) and spread onto LB plates. Antibiotic-containing filter discs were deposited onto the agar and plates were visualized through a blue-light lamp after 24 h of incubation at 37°C. Discs with mitomycin-C (10 μg), a known inducer of the SOS system, or without antibiotic were used as a positive or negative control, respectively.

Effect of antibiotics on cell morphology

The effect of low concentrations of antibiotics on cell morphology was studied by direct observation of the treated cultures under an Olympus BX61 microscope. Aliquots (2 mL) of exponentially growing ME12 cells (~10^8 cells/mL) were incubated with different antibiotics for 4 h at 37°C with shaking (250 rpm). After 4 h of treatment, 2 μL from each culture was used to prepare samples. These samples were scanned and photographed under the microscope with a UplanF1 100× NA 1.30 oil immersion objective.

Statistical analysis

Statistical evaluation was done by using the Mann–Whitney *U*-test when two groups were compared. Differences were considered significant when *P* values were $\leq$0.05.

Results

Effect of different concentrations of antimicrobials on E. coli mutagenesis

In principle, mutagenic activity of antimicrobials is expected to occur within a window of concentrations very close to the MIC (peri-MIC), because higher concentrations will kill or stop the growth of most of the cells in the population and lower ones will not have a stimulatory effect.[27] In this work, we investigated the mutagenic effect of 13 antimicrobials at peri-MIC concentrations on the strain ME12, an MG1655 derivative, by evaluating the appearance of mutants resistant to rifampicin or fosfomycin. We used the strain ME12 for consistency, because it was used to study the effect of the same antibiotics on homologous recombination.[5,6] This strain shows a spontaneous frequency of rifampicin-resistant mutants of 2×10^{-7} and of fosfomycin-resistant mutants of 1×10^{-6} (not shown). Table 1 shows the MIC of each antimicrobial under our experimental conditions for the strain ME12. The mutagenic effect was tested for five different concentrations, including two lower and two higher than the MIC and the MIC (i.e. 1/4× MIC, 1/2× MIC, MIC, 2× MIC and 4× MIC). The concentration of each antimicrobial producing the highest effect was re-tested using five independent replicates to confirm the results. Ten antimicrobials (ampicillin, ceftazidime, imipenem, fosfomycin, ciprofloxacin, trimethoprim, sulfamethoxazole, trimethoprim/sulfamethoxazole, colistin and tetracycline) produced statistically significant increases ($P\leq0.05$) in mutant frequency when it was calculated for rifampicin resistance, with maximal increases of 3.4-, 2.2-, 3.0-, 5.0-, 2.0-, 17.1, 6,3-, 8.7-, 3.0- and 2.1-fold, respectively (Figure 1a, black bars). The results from the other three drugs were not statistically significant ($P>0.05$). When the mutagenic effect was studied calculating the mutant frequency for fosfomycin resistance, eight antimicrobials (ampicillin, ceftazidime, imipenem, ciprofloxacin, trimethoprim, sulfamethoxazole, trimethoprim/sulfamethoxazole and tetracycline) produced statistically significant increases ($P\leq0.05$) in mutant frequency, with maximal

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Table 1. MICs (mg/L) of the antimicrobials used in this study for the wild-type strain ME12 and its *recA* derivative

Antibiotic	ME12	ME12△*recA*
Ampicillin	1	1
Ceftazidime	0.25	0.12
Imipenem	0.12	0.12
Fosfomycin	0.06	0.03
Ciprofloxacin	0.12	0.007
Trimethoprim	0.5	0.25
Sulfamethoxazole	256	256
Trimethoprim/sulfamethoxazole[a]	0.5/9.5	0.25/4.75
Colistin	8	2
Tetracycline	0.5	0.5
Gentamicin	0.5	0.5
Rifampicin	2	2
Chloramphenicol	2	2

[a]The proportions of trimethoprim and sulfamethoxazole in trimethoprim/sulfamethoxazole are 1:19, as indicated by EUCAST (http://eucast.www137.server1.mensemedia.net/clinical_breakpoints).

increases of 3.6-, 2.0-, 2.2-, 2.2-, 7.7-, 4.9, 7.9- and 3.0-fold, respectively (Figure 1b, black bars). The results from the other five drugs were not statistically significant ($P > 0.05$). Taken together, these results indicate that at least 8 out of 13 antimicrobials or combinations (those with positive results in both tests) produced increased mutagenesis levels at concentrations close to their MICs. Interestingly, while most antimicrobials produced mild increases in mutagenesis, trimethoprim, sulfamethoxazole and trimethoprim/sulfamethoxazole produced the highest increases in mutant frequency in both tests (rifampicin and fosfomycin resistance). As stated in the Materials and methods section, the resolution of filaments was verified by direct observation of samples from the different cultures under the microscope before plating, and only cultures with a proportion of filaments of <5% of total cells were plated. Thus, increased mutant frequency is not attributable to the presence of filamented cells in the treated cultures. A description of the effect of antibiotic treatment on cell morphology can be found below.

As the number of viable bacteria in the inoculum (after antibiotic treatment) might affect the observed frequency of mutants, we performed experiments with different inoculum sizes of untreated ME12 cells, ranging from 10^7 to 10^9 cells. No differences were observed in mutant frequency between these cultures (not shown). Thus, the final number of viable cells after treatment with different drugs was not the cause of the observed antibiotic-mediated stimulation of mutagenesis.

Finally and remarkably, treatment with rifampicin or fosfomycin did not produce an increased number of rifampicin- or fosfomycin-resistant mutants, respectively, thus indicating that the concentrations of these antibiotics and/or the time of exposure used in our experiments were not able to select for rifampicin- or fosfomycin-resistant variants.

SOS induction by different antimicrobials

The induction of the SOS stress response by some antimicrobials has been previously described.[1,2,7,28,29] To determine whether the observed stimulation of mutagenesis could be linked to SOS induction, we studied the effect of these antimicrobials on the induction of *recA* transcription. We used the disc-plate assay described in the Materials and methods section. Figure 2 shows that ampicillin, ceftazidime, ciprofloxacin, trimethoprim, sulfamethoxazole and trimethoprim/sulfamethoxazole induce transcription of the *recA*::GFP fusion, with the highest induction produced by ciprofloxacin, trimethoprim and trimethoprim/sulfamethoxazole.

Effect of RecA on antibiotic-mediated stimulation of mutagenesis

It has been demonstrated that ciprofloxacin and ceftazidime stimulate mutagenesis in *E. coli* through the induction of mutagenic DNA polymerases of the SOS system.[1,2] Consequently, we decided to study the effect of the different antimicrobials on mutant frequency in a *recA*-deficient background. As in the case of the wild-type strain, MICs of the different drugs were obtained for the *recA* strain (Table 1). As expected, a strong decrease in ciprofloxacin MIC was observed between the wild-type and its *recA*-deficient derivative. Also, small though consistent decreases were observed in the MICs of ceftazidime, fosfomycin, trimethoprim, trimethoprim/sulfamethoxazole and colistin.

The effects of drugs on the mutant frequency of the *recA*-deficient derivative were studied with different peri-MIC concentrations, including the MIC itself. The mutagenesis stimulated by ampicillin, imipenem, ciprofloxacin, trimethoprim, trimethoprim/sulfamethoxazole and tetracycline is abolished in the *recA* background (Figure 1a and b). Grey bars show the results with concentrations equivalent to the most mutagenic in the *recA*-proficient strain. None of the concentrations tested showed increased mutagenesis in the *recA*-deficient strain (except ceftazidime in the rifampicin-resistance test). Therefore, RecA is absolutely necessary for the stimulation of mutagenesis by the eight antimicrobials with positive results in both rifampicin- and fosfomycin-resistance tests.

Effect of mutagenesis-stimulating concentrations of antimicrobials on cell morphology

The effect of peri-MIC concentrations of antimicrobials on cell morphology (only the most stimulating concentrations are shown in Figure 3) were studied. Figure 3(a) shows that ampicillin, ciprofloxacin, trimethoprim, sulfamethoxazole and trimethoprim/sulfamethoxazole, and the SOS inducer mitomycin-C (positive control) produced, as expected, a clear filamentation of ME12 cells after 4 h of treatment. A slight cell enlargement can be seen with tetracycline. In addition, imipenem produced classical ball-shaped cells. We also studied the effect of the corresponding antimicrobial concentrations (see above) on the *recA*-deficient strain. Figure 3(b) shows that, as predicted from its mechanism of action (inhibition of the septation process via protein PBP3), ceftazidime also produced filaments in the *recA* mutant. Amazingly, ciprofloxacin and trimethoprim (and sulfamethoxazole to a lesser extent) produced filaments in the *recA* derivative, although shorter than in the wild-type. This is an unexpected result as the production of filaments by these antibiotics was believed to be caused by induction of the SOS

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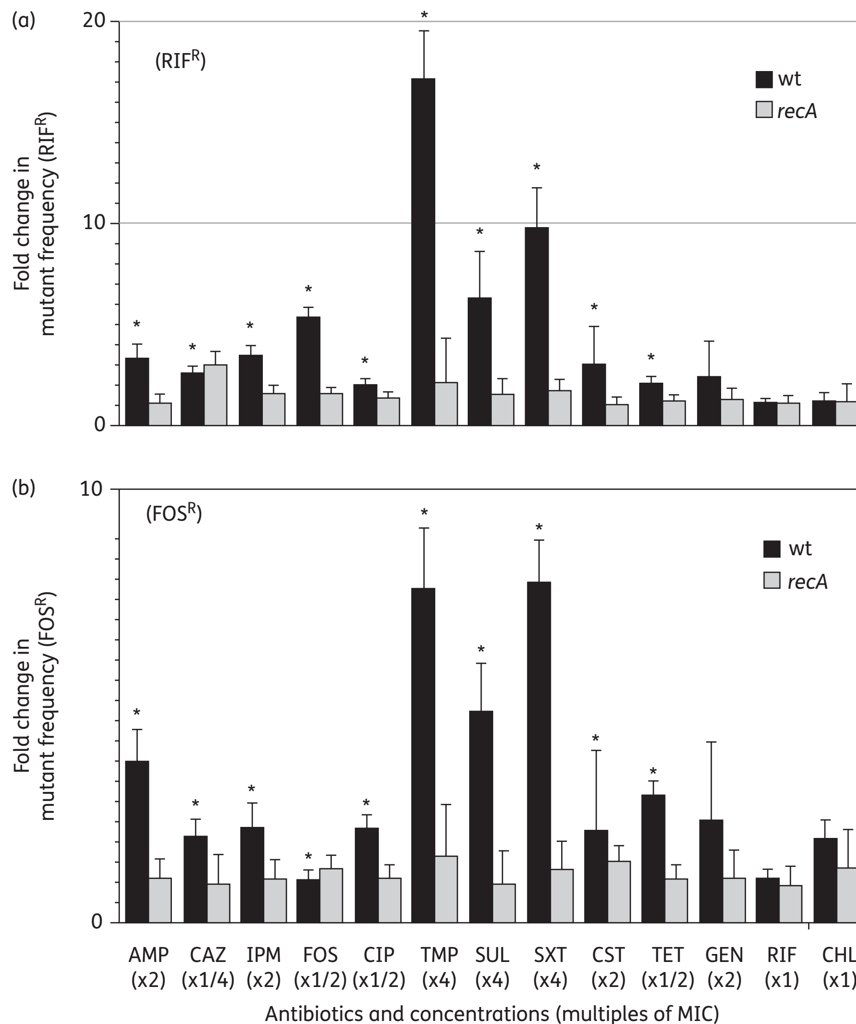

Figure 1. Effect of sublethal concentrations of antimicrobials on mutant frequency. Fold changes in mutant frequency of the wild-type (wt) strain ME12 (black bars) and its *recA* mutant derivative (grey bars) for rifampicin resistance (RIF^R) (a) and fosfomycin resistance (FOS^R) (b), after treatment with antibiotics. Data are relative to untreated controls (no antibiotic). Only concentrations with the highest change in mutant frequency are represented. The number in parentheses below the antibiotic indicates the concentration relative to its MIC. Values are the means of five experiments ± SD. Asterisks indicate that the fold increases relative to the untreated strain are statistically significant ($P \leq 0.05$, according to the Mann–Whitney *U*-test). AMP, ampicillin; CAZ, ceftazidime; IPM, imipenem; CIP, ciprofloxacin; TMP, trimethoprim; SUL, sulfamethoxazole; SXT, trimethoprim/sulfamethoxazole; CST, colistin; TET, tetracycline; GEN, gentamicin; CHL, chloramphenicol.

system (see below). Thus, according to our results, a *recA*-independent mechanism of filamentation can be predicted.

Discussion

The extended use of antimicrobial drugs over the past six decades has had a major impact on human-associated bacteria (both commensals and pathogens), leading to the selection and spread of resistant variants. The exposure of bacteria to antibacterial agents results in the selection of pre-existing resistant variants that ultimately become fixed in the population.[30–32] Antibiotic pressure may also select for bacteria with an increased mutation rate (hypermutators),[33] increasing the probability of bacteria becoming resistant.

Induction of the SOS response by antimicrobials such as fluoroquinolones, and increased mutagenesis as a consequence was described a long time ago (see Ysern *et al.*[2] and references therein). Because the SOS response is efficiently activated by DNA-damaging agents and this activation leads to the transcriptional induction of error-prone DNA polymerases, it is not surprising that clear evidence of antimicrobial-induced mutagenesis was first described with fluoroquinolones.[2,34] However, induction of the SOS response, and its resultant increased mutagenesis, by β-lactams was described many years later.[1,28]

A recent article has demonstrated that some antimicrobials, defined as bactericidal, such as ampicillin, norfloxacin and kanamycin, stimulate the production of highly deleterious ROS radicals in Gram-negative and Gram-positive bacteria, which

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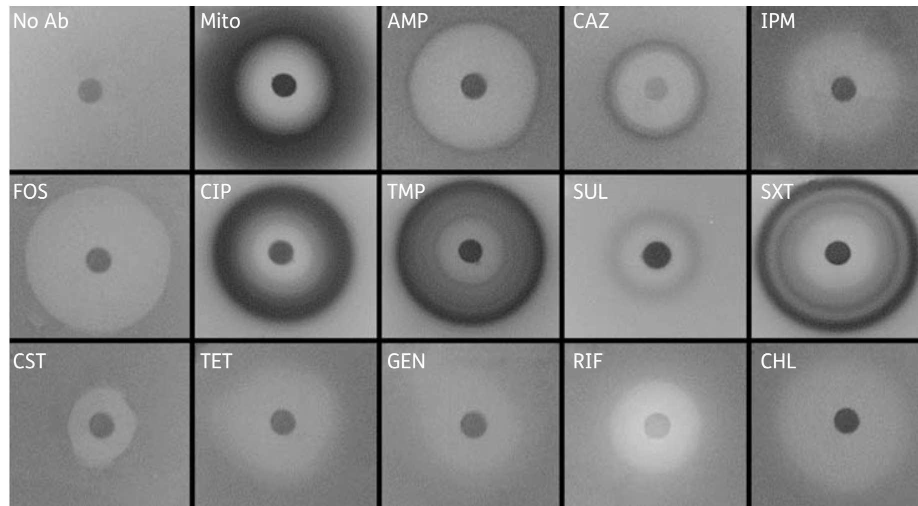

Figure 2. Effect of antibiotics on the transcription of the *recA*::GFP fusion on a solid surface. An aliquot of 100 μL of an overnight culture was inoculated into LB soft agar (0.7% agar) and spread onto LB plates. Antibiotic-containing filter discs were deposited onto the gelified agar, and plates were visualized under a blue-light lamp after 16 h of incubation at 37°C. Induction is observed as a fluorescent band around the inhibition halo produced by ampicillin, ceftazidime, ciprofloxacin, trimethoprim, sulfamethoxazole and trimethoprim/sulfamethoxazole. This assay permits the exploration of the full range of antibiotic concentrations for SOS induction without knowing the most effective one. The discs contain different amounts of antibiotic: ampicillin (AMP; 500 μg); ceftazidime (CAZ; 10 μg); imipenem (IPM; 40 μg); fosfomycin (FOS; 100 μg); ciprofloxacin (CIP; 10 μg); trimethoprim (TMP; 300 μg); sulfamethoxazole (SUL; 3200 μg); trimethoprim/sulfamethoxazole (SXT; 13.5/256 μg); colistin (CST; 200 μg); tetracycline (TET; 100 μg); gentamicin (GEN; 200 μg); rifampicin (RIF; 200 μg); and chloramphenicol (CHL; 300 μg). Discs containing no antibiotic (No Ab) or 10 μg of mitomycin-C (Mito), a known inducer of the SOS system, were used as negative and positive controls, respectively.

ultimately can contribute to cell death.[7] In contrast, bacteriostatic drugs do not produce such an effect. Interestingly, the cited article also shows that the same bactericidal drugs induce the SOS stress response and that inactivation of RecA, a co-regulator of the response, produces increased susceptibility to some of them, including norfloxacin, ampicillin and kanamycin.[7,35] Our results show that mutagenesis is induced not only by bactericidal drugs but also by bacteriostatic ones, and that inactivation of RecA activity abolishes the induction of mutagenesis in all cases.

An interesting result from our investigation is that trimethoprim alone, but also in combination with sulfamethoxazole, promotes the highest increase in mutant frequency (Figure 1a and b). Trimethoprim prevents incorporation of thymine into bacterial DNA by inhibition of dihydrofolate reductase.[36] This provokes not only the induction of the SOS response,[37] but also a nucleotide pool imbalance that can affect replication fidelity.[38,39] Therefore, both SOS response and nucleotide imbalance might act synergistically to increase mutant frequency. Another interesting result is the production of filaments in the *recA* derivative by ciprofloxacin and trimethoprim treatments. The production of filaments by these antibiotics was believed to be caused by the SOS response, which is mediated by RecA and LexA. This response induces the transcription of the *sulA* (*sfiA*) gene.[40] SulA interacts reversibly with the protein FtsZ,[41] causing inhibition of cell division and consequent filamentation. *sulA*-independent filamentation is also known in *E. coli*, although this mechanism is also dependent on SOS induction.[42] Our results suggest that a new SOS-independent mechanism mediates filamentation in the absence of RecA. In fact, we have detected that, at least for ciprofloxacin, filamentation occurs in a *sulA*-deficient background (not shown).

We show here that a number of antibiotics can increase genetic variation by the stimulation of mutagenesis in treated bacteria, suggesting that antibiotic treatments may favour the acquisition and/or evolution of some mechanisms of antibiotic resistance. For instance, some extended-spectrum β-lactamases are the result of combining a reduced number of mutations.[43–46] Thus, sublethal concentrations of mutagenic antimicrobials (not necessarily β-lactams) may accelerate the evolution of new extended-spectrum variants by stimulating the production and accumulation of mutations. Another example is the resistance conferred by increased expression of efflux pumps. It can occur via mutation in different targets, including mutations in the local repressor gene, mutation in a non-related global regulatory gene and changes in the promoter region of the efflux-pump gene (see for instance Piddock[47]).

The stimulatory effect on mutagenesis described here for some of the tested antimicrobials is very low, and may be considered too modest to exert any effect on bacterial evolution. However, it has been stated that modest changes in mutation rate can greatly influence antibiotic resistance development.[48] As concerns the possibility of finding stimulatory concentrations by a sufficiently dense bacterial population, we have to consider the vast numbers of bacteria challenged by antibiotic treatments. Antibiotics are mainly used to combat pathogens but they also challenge commensals collaterally. While an infection is usually produced by a relatively small number of cells (10^8–10^9), ~10^{14} prokaryotic cells from hundreds of different species form our commensal flora,[49] with different intrinsic levels of antibiotic susceptibility. Finally, even resistant microorganisms might be included among the possible targets for the mutagenic effect of antibiotics as high concentrations of antibiotics must be considered sublethal

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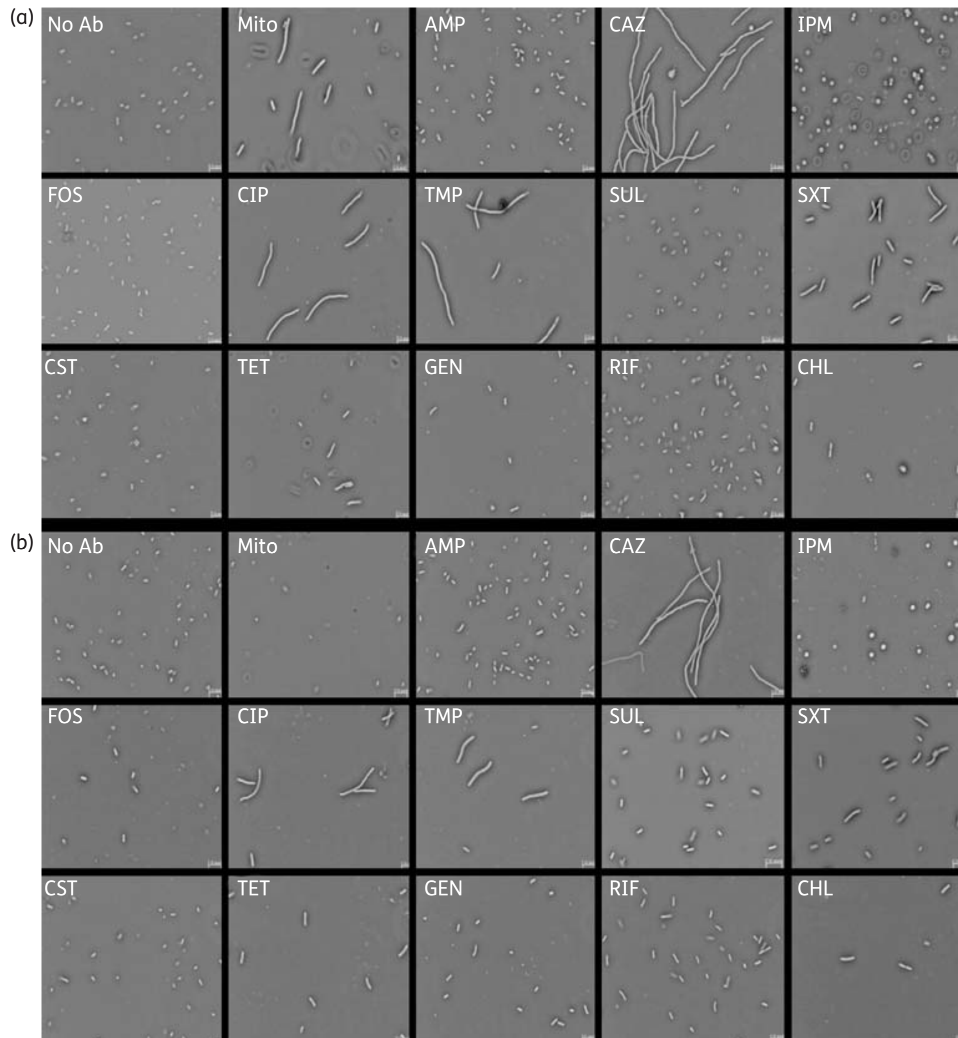

Figure 3. Effect of antibiotics on cell morphology. Bacterial cultures were treated for 4 h as indicated in the Materials and methods section. (a) Effect on the wild-type strain ME12. (b) Effect on the ME12 *recA* derivative. Bars at the right bottom of each image represent 10 μm. No Ab, no antibiotic; Mito, mitomycin-C; AMP, ampicillin; CAZ, ceftazidime; IPM, imipenem; FOS, fosfomycin; CIP, ciprofloxacin; TMP, trimethoprim; SUL, sulfamethoxazole; SXT, trimethoprim/sulfamethoxazole; CST, colistin; TET, tetracycline; GEN, gentamicin; RIF, rifampicin; CHL, chloramphenicol.

for resistant bacteria. Thus, any particular window of sub-MIC mutation-stimulating concentrations of antibiotics should not be difficult to find. The fact that thousands of tons of antibiotics are used every year to treat billions of human and veterinary infections and to promote animal growth increases the probability of finding suitable conditions for the stimulation of mutagenesis.

Studies by Romesberg and coworkers[8,9] have shown that prevention of SOS activation resulted in a decrease in both survival and mutagenesis in ciprofloxacin-treated cultures, as well as ciprofloxacin or rifampicin-treated infected mice. Consequently, the possibility that components of induced mutation pathways might be inhibited as a novel therapeutic strategy to prevent the development of antibiotic resistance has been proposed.[50] Efforts have been made to identify small molecules and short peptide inhibitors of RecA activity,[51–53] although the absence of potential adverse effects on Rad51 (the human RecA homologue) needs to be demonstrated.

Our study aimed to explore the effect of RecA inhibition on induced mutagenesis produced by many antibiotics. Our results with the *recA*-defective strain suggest that most, if not all, mutagenesis induced by sublethal concentrations of antibiotics is dependent, directly or indirectly, on RecA activity, thus supporting the hypothesis that inhibition of RecA is a plausible therapeutic adjuvant in combined therapy to reduce the capacity to generate antibiotic-resistant mutants, with the additional advantages of affecting susceptibility, homologous recombination,[20] swarming motility[21] and biofilms.[22]

Funding

This work was supported by the Ministerio de Ciencia e Innovación, Instituto de Salud Carlos III (ISCIII) co-financed by the European Development Regional Fund 'A way to achieve Europe' ERDF, the Spanish Network for Research in Infectious Diseases (REIPI RD06/0008) and

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grant PI070215 from ISCIII and the European Community (PAR, FP7-HEALTH-2009-241476). T. D. T. is the recipient of a JAE predoc fellowship from the CSIC.

Transparency declarations

None to declare.

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2. El gen *dinF* de la respuesta SOS protege frente a estrés oxidativo y sales biliares

Los primeros estudios de sobre el sistema SOS, poco después de que dicha denominación fuese acuñada (154), fueron realizados, entre otros, en el laboratorio de Graham C. Walker al principio de la década de los ochenta. Mediante un elegante sistema basado en un transposón que generaba aleatoriamente fusiones transcripcionales Mu*d*1-*lacZ* identificaron un grupo de cinco genes que se inducían en respuesta al daño en el DNA de manera *lexA* y *recA* dependiente (129). Entre aquellos genes, que fueron denominados genes *din* (*DNA-damage inducible*), se encontraba el protagonista de esta sección: *dinF*. Un estudio posterior del mismo grupo demostró que se encontraba formando un operón con *lexA* (155).

Una localización genómica tan notable, junto con la aristocracia reguladora de la respuesta SOS, apunta a una regulación exquisita y por tanto a un papel tan relevante como intrigante para *dinF*. Sin embargo, durante años, ningún estudio en *E. coli* volvió a arrojar luz sobre la o las posibles funciones de dicho gen.

Dos factores nos llevaron a emprender la caracterización del papel fisiológico de *dinF*. El primero de ellos, que acabó por ser determinante, es que nuestro grupo había estudiado y caracterizado previamente la función de NorM, una bomba de expulsión que está relacionada con la protección frente a peróxido de hidrógeno y que pertenece a la familia MATE (*multridrug and toxic compound extrusion*) (156). Por homología de secuencia se había descrito que DinF es un miembro prototípico de esta familia (157). Además, un análisis en BLAST demostró una similitud notable entre ambas proteínas, sugiriendo la posibilidad de que tuviesen una función común. El segundo factor es la ya mencionada regulación de *dinF* por el sistema SOS, uno de los hilos conductores de la presente tesis. ¿Sería *dinF* una bomba de expulsión controlada por la respuesta SOS, capaz de paliar los daños producidos por el estrés oxidativo?

Efectivamente, los resultados obtenidos sugieren que DinF, al igual que NorM, es capaz de proteger a la célula frente al daño oxidativo aumentando la viabilidad y reduciendo los radicales libres intracelulares y la carbonilación proteica tras un tratamiento con peróxido de hidrógeno. Asimismo, DinF es capaz de reducir la mutagénesis espontánea en un fondo carente del gen *mutT*, uno de los responsables de lidiar con la incorporación de 8-oxo-guanina en el DNA. Este nucleótido es el causante de la mayor parte de las mutaciones producidas por daño oxidativo (11). Además DinF confiere una ventaja adaptativa en presencia de sales biliares, un agente oxidante y genotóxico al que las enterobacterias se ven sometidas regularmente en el tracto digestivo.

A pesar de nuestros esfuerzos, la naturaleza del sustrato o sustratos que expulsa o introduce DinF permanece aún oculta. No obstante, quizás la mayor virtud de este trabajo sea que se trata de la primera caracterización de una bomba de membrana controlada por el regulón SOS, sugiriendo la existencia de una estrategia celular para expulsar agentes tóxicos antes de que causen daños mayores.

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PLoS one

The *Escherichia coli* SOS Gene *dinF* Protects against Oxidative Stress and Bile Salts

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Abstract

DNA is constantly damaged by physical and chemical factors, including reactive oxygen species (ROS), such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH). Specific mechanisms to protect and repair DNA lesions produced by ROS have been developed in living beings. In *Escherichia coli* the SOS system, an inducible response activated to rescue cells from severe DNA damage, is a network that regulates the expression of more than 40 genes in response to this damage, many of them playing important roles in DNA damage tolerance mechanisms. Although the function of most of these genes has been elucidated, the activity of some others, such as *dinF*, remains unknown. The DinF deduced polypeptide sequence shows a high homology with membrane proteins of the multidrug and toxic compound extrusion (MATE) family. We describe here that expression of *dinF* protects against bile salts, probably by decreasing the effects of ROS, which is consistent with the observed decrease in H_2O_2-killing and protein carbonylation. These results, together with its ability to decrease the level of intracellular ROS, suggests that DinF can detoxify, either direct or indirectly, oxidizing molecules that can damage DNA and proteins from both the bacterial metabolism and the environment. Although the exact mechanism of DinF activity remains to be identified, we describe for the first time a role for *dinF*.

Citation: Rodríguez-Beltrán J, Rodríguez-Rojas A, Guelfo JR, Couce A, Blázquez J (2012) The *Escherichia coli* SOS Gene *dinF* Protects against Oxidative Stress and Bile Salts. PLoS ONE 7(4): e34791. doi:10.1371/journal.pone.0034791

Editor: Floyd Romesberg, The Scripps Research Institute, United States of America

Received July 24, 2011; **Accepted** March 9, 2012; **Published** April 16, 2012

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Funding: This work was supported by grants PI10/00105 (FIS) and REIPI (RD06/0008), both from Ministerio de Ciencia e Innovación, Instituto de Salud Carlos III, the last co-financed by European Development Regional Fund "A way to achieve Europe" ERDF, Spanish Network for Research in Infectious Diseases, and by the PAR project (Ref 241476) from the EU 7th Framework Programme. JRG was the recipient of a FPI fellowship from the Spanish Ministry of Science and Innovation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Oxidative stress, the inevitable consequence of living in an oxygen-rich environment, occurs when the cellular redox balance is upset by increased doses of reactive oxygen species (ROS). Microorganisms living in aerobic environments are constantly exposed to ROS, which are generated by the aerobic metabolism and environmental agents. ROS, including superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH), are highly reactive molecules that can damage key cellular components, including DNA, proteins, carbohydrates and lipids. To defend themselves against ROS injuries, microorganisms have developed different constitutive and inducible mechanisms, including scavenging systems, like superoxide dismutases (SOD) and catalases/peroxidases, export of redox-cycling substances, like the AcrAB-TolC efflux pump, and DNA repair systems, like DNA-glycosilases [1]. In addition, commensal and pathogenic bacteria have to cope with oxidative responses from the host, such as bile salts in the gastrointestinal tract or H_2O_2 from phagocytes.

Specific mechanisms to protect and repair DNA lesions produced by reactive forms of oxygen have been developed. When DNA lesions are persistent the SOS system, an inducible response, is activated to rescue cells from severe DNA-damage [1]. In *Escherichia coli* the expression of more than 40 genes [2], many of them playing key roles in DNA damage tolerance mechanisms, is regulated by the LexA repressor [3], which autogenously regulates its own transcription [4]. When no DNA damage occurs, the cellular levels of LexA repressor suffice to repress the system. The blockage of DNA replication originated by DNA damage, including that produced by ROS, generates stalled replication forks and, consequently, single stranded DNA (ssDNA) [1]. This ssDNA is the molecular distress signal allowing the nucleation of RecA monomer protein around it. The interaction ssDNA-RecA produces the RecA* coprotease activity, which promotes the autocleavage of the LexA repressor. This process decreases the intracellular level of LexA, which in turn releases the repression of SOS genes, switching on the system. DNA repair functions, such as excision repair (UvrABC), Holliday resolution junctions (RuvAB), and translesion synthesis (TLS) polymerases, are SOS-induced [1]. Finally, when the distress signal disappears, the level of RecA* decreases and that of LexA repressor increases, leading the SOS system to the repressed state.

By generating random Mu*d*1-*lacZ* transcriptional fusions Kenyon and Walker [5] identified a set of damage inducible (*din*) genes whose expression was increased by different SOS-inducing treatments. The function of many of these *din*, and other SOS, genes has been identified (for a review see [1]). However, the role of some, such as *dinF*, remains unknown. By sequence homology it has been deduced that *dinF* encodes a 49 kDa

multidrug and toxic compound extrusion (MATE) family membrane protein [6,7]. Members of the MATE family of transporters characteristically possess 12 putative transmembrane domains and have been found in all three domains of life, including humans, where they mediate the efflux of organic cations using the transmembrane proton gradient as a driving force [6,7].

Recently, we have shown that expression of the *E. coli* MATE protein NorM protects the cells from the H_2O_2 killing effect, particularly when other protective mechanisms are absent [8]. These results led us to test whether the expression of DinF could also diminish the H_2O_2 lethality. We have also analyzed different effects (intracellular ROS levels, protein carbonylation, antibiotic resistance, and mutation rate) produced by the expression of *dinF* in different genetic backgrounds. Because in *E. coli dinF* forms an operon with *lexA,* the master regulator of the SOS response, we have studied the putative co-regulation of *lexA* and *dinF* transcription in all sequenced bacterial genomes. Finally, as both genes appear to form a single operon only in *Enterobacteria*, we have analyzed whether *dinF* protects from bile salts, a known oxidant product present in the gastrointestinal tract [9].

Materials and Methods

Bacterial strains and plasmids

The *E. coli* strain NR10831 [F′CC101] (*ara, thi, rif*^{R}, *nal*^{R}, *metB,* Δ*prolac*) was a gift from I. Fijalkowska (Institute of Biochemistry and Biophysics, Warsaw, Poland). The F′CC101 carries an specific *lacZ* mutation affecting residue Glu-461 in β-galactosidase. Only an AT to CG base substitution will restore the glutamic acid codon and the Lac$^+$ phenotype [10]. The strains NR10831 Δ*dinF* and NR10831 Δ*mutT* were constructed by P1 transduction of the Δ*dinF*::Kan and Δ*mutT*::Kan alleles from the corresponding strains of the Keio Collection [11] as described [12]. Because of NR10831 is resistant to rifampicin, the *E. coli* BW25113 strain and its mutant derivatives (also constructed by P1 transduction) were used to estimate mutation rates to rifampicin resistance. To discard that the *dinF* deletion could alter the *lexA* regulation/ expression and that of other *lexA*-regulated genes, the expression of both *recA*::GFP and *lexA*::GFP transcriptional fusions [13] were studied under SOS induced and non-induced conditions. No differences were found between wild-type and *dinF*-defficient strains (data not shown).

The vector pCA24N [14] and its derivative pDinF, containing the wild-type *dinF* gene, were obtained from the Complete Set of *E. coli* K-12 Open Reading Frame Archive (ASKA) library [14].

Materials and media

The bacteria were grown in liquid M9 minimal medium with 1% glucose and a mixture of amino acids (10 μg/ml each) or LB. The following materials were obtained from Sigma: IPTG (isopropyl-β-D-thiogalactopyranoside), chloramphenicol, ripampicin, trizma-base, sodium dodecyl sulphate (SDS), DNAse, RNAse, norfloxacin, ofloxacin, streptomycin, mitomycin C and ethidium bromide. We also used the following materials: lysozyme and glycerol (United States Biochemical Corporation), ampicillin (Biochemie GmbH) and bile salts (N° 3, Pronadisa, Spain), ciprofloxacin and gentamicin (Normon SA, Spain), ceftazidime (Combino Pharm), kanamycin (Q-biogene, USA) and H_2O_2 (FMC Foret, Spain). Carbonylated proteins were detected using the chemical and immunological reagents from the OxyBlot Oxidized Protein Detection Kit (Chemicon). Dihydrorhodamine 123 (DHR) for detection of ROS was from Enzo® Life Sciences.

Estimation of H_2O_2-induced cell death

Strains were grown at 37°C in M9 supplemented with appropriate antibiotics to mid-exponential phase and washed with 0.9% NaCl solution. Cells were treated with different concentrations of H_2O_2 (1, 12.5, 25 and 50 mM) for 30 min at 37°C and washed with 1 ml of 0.9% NaCl. A non-treated control was also included. Appropriate dilutions were immediately plated onto LB plates and incubated overnight at 37°C to determine viability. Experiments consisted of five independent cultures for each strain. Cell survival was calculated by comparing the number of colony forming units (cfu) of treated to those of the untreated cells.

Estimation of mutation rates

Lac reversion assays were carried out in M9 minimal medium. Inocula were grown with glucose as carbon source and plates were supplemented with lactose as unique carbon source according to Miller [12]. The scavenger strain MEC222 [15], harboring a truncated *lacZ* allele (*lacZ*ΔT::*cat*) with the c-terminal region replaced by the *cat* cassette, was added to the lactose agar MM before being spread (40 μl of a stationary phase culture per litre of media, approximately 10^7 cells/plate). Plates were stored overnight at room temperature. The desired cultures for Lac reversion assays were spread onto a M9 top agar layer, without carbon source and supplemented with 5-bromo-4-chloro-3-indolyl-3-D-galactoside (X-Gal), as described by Miller [12].

To calculate the mutation rate, pre-inocula were initiated in tubes with 3 ml of M9 glucose directly from frozen samples. The pre-inocula were grown at 37°C overnight to the stationary phase. From each culture about 10^4 cells were inoculated in 40 ml of M9 glucose and divided into 4 independent cultures, 10 ml each and less than 10^4 cells/culture. These inocula were grown for 24 hours. Appropriate dilutions of the saturated cultures were plated onto selective medium Lac X-gal MM to determine the number of Lac$^+$ mutants, respectively. LB plates were used to determine the total cfu. To calculate the mutation rate to rifampicin resistance, the strain BW25113 and its mutant derivatives were inoculated in LB with appropriate antibiotics directly from frozen samples and incubated overnight. Less than 10^4 cells were inoculated in each one of 4 flasks containing 2 ml of LB and allowed to grow for 24 h at 37°C with strong shaking. Then, appropriate dilutions were plated onto LB-rifampicin (100 μg/ml) agar plates to select spontaneous resistant mutants. LB plates were used to estimate the number of viable cells in the culture. This protocol was performed by quadruplicate.

Mutation rates were calculated using the Ma-Sandri-Sarkar maximum likelihood (MSS-ML) method [16] as implemented by Falcor web tool [17,18].

Flow cytometry

Flow cytometry analysis was performed using the H_2O_2-activated fluorescent dye dihydrorhodamine 123 (DHR). DHR is a probe for detection of intracellular reactive oxygen species. It is oxidized into rhodamine 123 which produces a maximal emission at 529 nm when excited at 507 nm (Enzo® Life Sciences). Wild type and mutant derivatives were grown in M9-glucose at 37°C to mid exponential phase of growth. Cells (0.5 ml/culture) were pelleted by centrifugation, and resuspended in saline containing 15 μM DHR, and then incubated for 15 min and diluted 1:500 in phosphate-buffered saline. The fluorescence levels (excitation 488 nm and emission 530 nm) of 15,000 cells were then counted for each strain under each condition using a FACSCalibur cytometer (BD Biosciences). WinMDI (The Scripps Institute, Purdue University, USA) was used for data analysis. Values

obtained were the geometric mean of the fluorescence from the 15,000 cells. Experiments were performed three times.

Determination of the cellular level of protein carbonylation

Wild type and mutant derivatives were grown overnight and then each one was split into two cultures (one control and one treated with 50 mM H_2O_2) and incubated as described above for 30 min, and cultures were submitted to peroxide challenge. After this time, peroxide was removed by centrifugation. Then, cells were washed, resuspended in M9 medium preheated to 37°C and further incubated. Cells were lysed as follows: 1 ml of the culture was washed with 50 mM Tris buffer (pH 7.5) and centrifuged for 10 min at 14,000 rpm. The pellet was re-suspended in 150 µl lysis buffer containing 0.5 mg/ml lysozyme, 20 µg/ml DNAse, 50 µg/ml RNAse, 1 mM EDTA, and 10 mM Tris (pH 8). 15 µl of 10% SDS solution was added and the cells were incubated at 100°C for 5 min. To examine the level of protein carbonylation in these lysates, we used the Chemicon OxyBlot kit to derivatize the carbonyl groups in the protein side chains to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine. These DNP derivative crude protein extracts were dot blotted onto a nitrocellulose membrane, which was incubated with primary antibody, specific to the DNP moiety of the proteins, and subsequently incubated with secondary (goat anti-rabbit) horseradish peroxidase-antibody conjugate directed against the primary antibody. Carbonylation was observed by ECL (Amersham Pharmacia Biotec). The intensity of each dot was quantified by densitometry analysis using the Image Master VPS-CL. The intensity of each dot was normalized to equal levels of protein, which were determined using Bradford reagent (Bio-Rad) and expressed in femtomoles of DNP, according to the control of the OxyBlot kit. Assays were done by triplicate.

Determination of minimal inhibitory concentrations

Minimal inhibitory concentrations (MIC) of ciprofloxacin, norfloxacin, ofloxacin, ampicillin, ceftazidime, streptomycin, kanamycin, gentamicin, bile salts, H_2O_2, mitomycin C and ethidium bromide were determined for the strain NR10831 harbouring either the empty vector pCA24N or the plasmid expressing *dinF* (pDinF). MICs with these two strains were studied by adding IPTG (50 µM final concentration) to achieve maximal expression. MICs were determined by inoculating mid-log phase grown strains in the wells of a 96-microwell plate. The bacterial inoculum was prepared using the same procedure in all cases. Approximately 10^3 cells from overnight cultures were inoculated into tubes containing 10 ml of LB broth supplemented with appropriate antibiotics and IPTG. The tubes were incubated at 37°C with strong agitation until the mid-log phase of growth (approximately 10^8 cells/ml). Then, 2×10^4 to 4×10^4 cells from these cultures were inoculated into each microdilution well containing LB and doubling concentrations of the desired substance. Incubation was at 37°C for 24 h. The MIC was defined as the minimal concentration where no growth was observed. Four replicas were performed for each antibiotic and strain.

Competitions in bile salts

After plating in M9 with lactose as unique carbon source spontaneous Ara+ revertants were isolated for the strains NR10831 and NR10831 *ΔdinF*::Kan and were transformed subsequently with plasmids pCA24N and pDinF. Competition assays were performed as described previously [19]. Briefly, 100 µl of a 1:1 mixture of overnight cultures of each strain were inoculated into flasks containing 9.90 ml of fresh LB medium supplemented with different concentrations of bile salts (Bile salts n° 3, Pronadisa, Spain) and allowed to grow for 24 h at 37°C with strong shaking (250 rpm). In order to distinguish between strains, in all competitions one competitor was Ara+ and the other Ara-. Competitions were repeated reversing the marker (competitor one Ara+ and competitor two Ara- and vice versa) to ensure that the Ara mutation has no effect on fitness determination. Initial (N_0) and final (N_t) densities of each strain were estimated by plating appropriate dilutions on LB and M9 with lactose as unique carbon source agar plates. Relative fitness was calculated as the ratio of growth rates (r) of each strain or $W = r_{\Delta dinF}/r_{wt}$ where $r = \ln N_t/N_0$. Results given are the mean fitness of eight replicates.

lexA-dinF operon in bacterial genomes

To study the putative co-regulation of *lexA* and *dinF*, we performed a search in operonDB (http://operondb.cbcb.umd.edu) for the *E. coli* MG1655 genome. Then, we selected the *lexA-dinF* operon and analysed the co-occurrence of that operon in all bacterial genomes stored in the database.

Results

Search of NorM homologues in *E. coli*

DinF and NorM from *E. coli* K12 show a 20% identity and a 36% similarity [20] according to a Blast search done at the NCBI site (http://ncbi.ace.uk) (Fig. 1). DinF contains, like NorM, twelve predicted transmembrane domains (Fig. 1). Therefore, in principle, a similar activity can be expected for both of them.

H_2O_2-induced killing

Previous results from our laboratory showed that the expression of *norM* reduced the H_2O_2-induced killing [8]. Consequently, we tested whether the expression of *dinF* could also decrease the killing by H_2O_2. Figure 2 shows that sensitivity to H_2O_2-mediated killing of cells lacking *dinF* (Δ*dinF*::Kan) is clearly higher than that of the wild type cells. The multicopy expression of *dinF* (pDinF) rescues the wild type survival level. To know how much dinF is over-expressed in the plasmid, we performed RT-qPCR of *dinF* in both NR10831 and NR10831 Δ*dinF*::Kan (pDinF). Expression of *gapA*, which encodes the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) enzyme, was used as an endogenous reference [21]. The results show that *dinF* expression is about 18 times higher in the plasmid than in the chromosome (data not shown).

Expression of *dinF* protects from H_2O_2-induced killing in the absence of MutT activity

Oxidation of guanine to 7,8-dihydro-8-oxo-guanine (8-oxoGTP) is especially noteworthy because it is highly mutagenic. *E. coli* possesses an efficient system to reduce the mutagenic effects of 8-oxoG, the GO repair system, consisting in three proteins, MutM, MutY and MutT [22]. MutT is a nucleoside triphosphate pyrophosphohydrolase, which converts 8-oxodGTP to 8-oxodGMP and pyrophosphate, inactivating this mutagenic activity. In the absence of MutT there is an increase in AT to CG mutations [22].

GO-deficient cells have been demonstrated to be more sensitive to H_2O_2-induced killing than those of the wild-type [8,23,24] *via* a still unknown mechanism. Interestingly, multicopy expression of *norM* was able to increase survival of *mutT*-deficient cells after exposure to H_2O_2 [8]. Figure 2 shows that multicopy expression of *dinF* promotes protection of *mutT*-deficient cells from H_2O_2-induced killing.

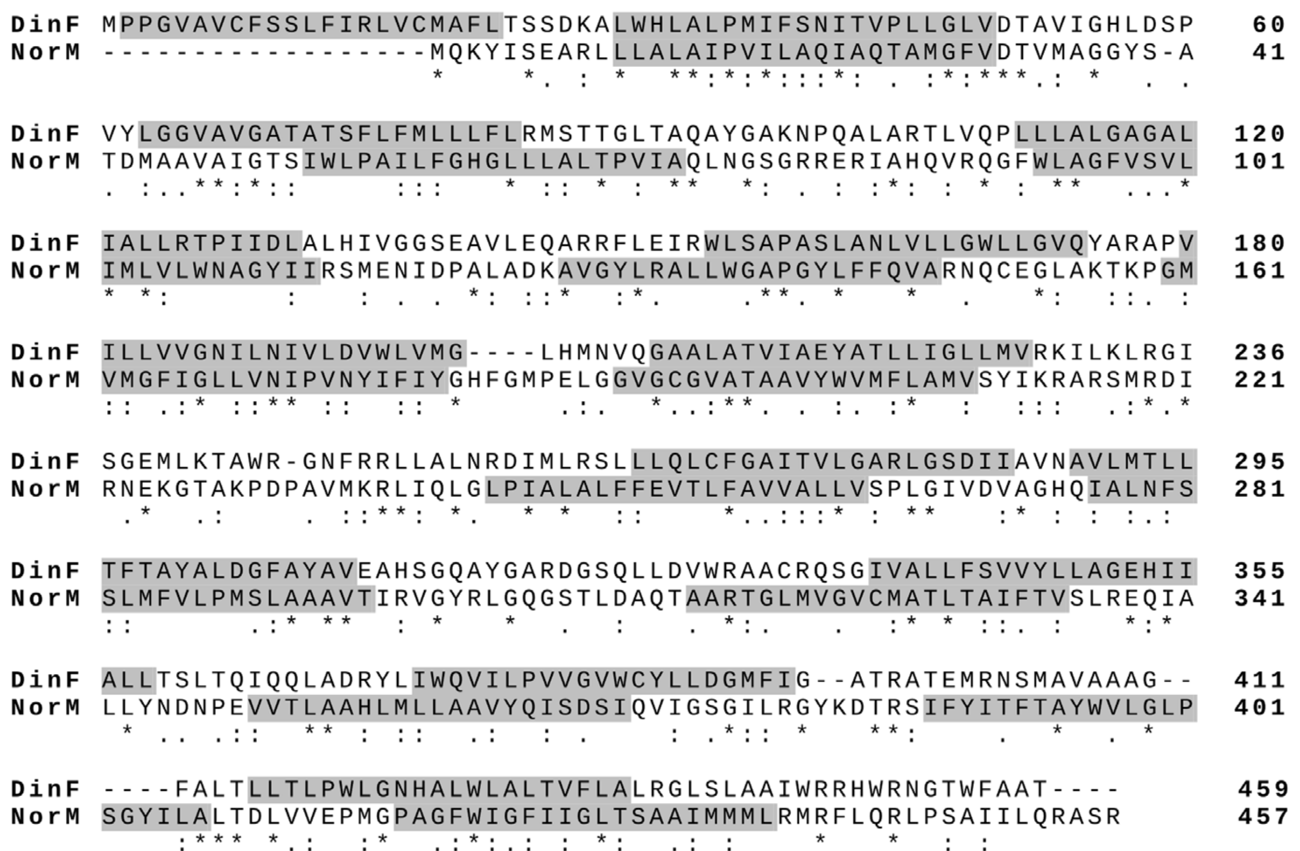

Figure 1. Sequence alignment of DinF and NorM. Aligment (ClustalW) of sequences from E. coli K12 was done according to Uniprot tools (http://www.uniprot.org) [42]. Predicted transmembrane domains are highlighted in grey.
doi:10.1371/journal.pone.0034791.g001

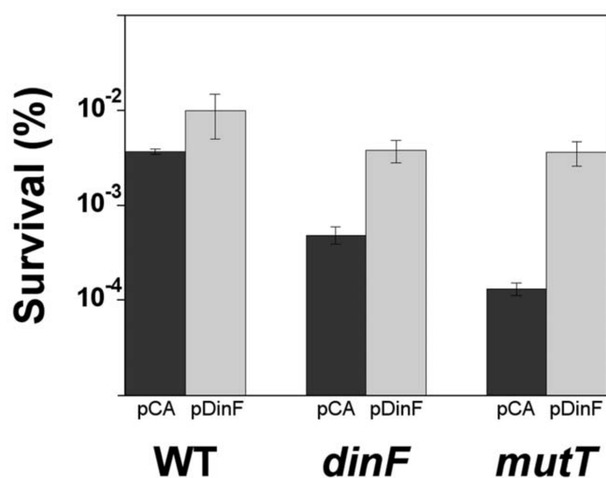

Figure 2. Viability after H_2O_2 treatment. The data represent survival percentages after 30 min of 50 mM H_2O_2 treatment. Shown are the strains NR10831 (WT) and its mutant derivatives Δ*dinF* and Δ*mutT* harboring the empty vector pCA24N (black) or the plasmid expressing *dinF*, pDinF (gray). The error bars indicate the standard deviation of four independent replicates.
doi:10.1371/journal.pone.0034791.g002

Overall, all these results demonstrate that DinF protects cells from H_2O_2-induced killing, mainly in the absence of *dinF* or *mutT*.

Effect of *dinF* expression on intracellular ROS levels

The above results suggest that DinF may control the level of intracellular ROS, which cause H_2O_2-induced killing. If this is true, the intracellular ROS levels should be diminished upon *dinF* expression. To assess this, the intracellular ROS levels in dihydrorhodamine 123 (DHR)-treated cells by flow cytometry were studied. DHR is a probe for the detection of intracellular reactive oxygen species. It is oxidized into rhodamine 123, which produces a maximal emission at 529 nm when excited at 507 nm (Enzo® Life Sciences). Figure 3 shows that the expression of *dinF* in the multicopy plasmid pDinF produces a slight but consistent decrease in the amount of intracellular ROS in both the wild type and Δ*dinF* strains. Figure 3 also shows that *dinF* expression produced a great decrease in intracellular ROS when expressed in a *mutT* background.

Effect of *dinF* expression on protein carbonylation

Carbonyl groups are introduced into protein side chains by site-specific oxidative modifications. Thus, carbonyl quantification provides an estimation of the oxidation status of proteins. The effect of *dinF* on protein carbonylation was studied in non-treated cells. The level of spontaneous protein carbonylation in the wild type, Δ*mutT* and Δ*dinF* strains growing in exponential phase was undetectable with the OxyBlot kit. However, when submitted to

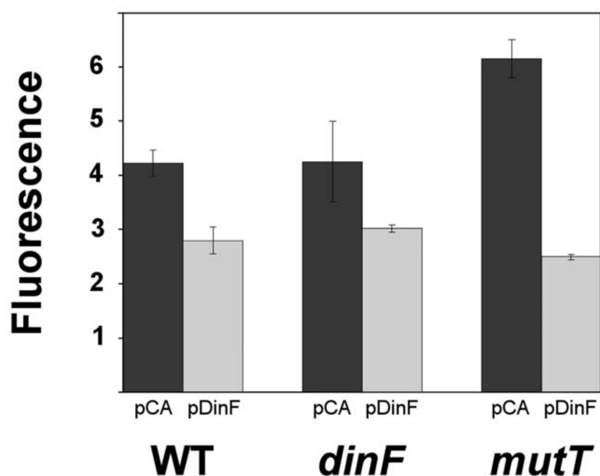

Figure 3. ROS levels in the *E. coli* wild type, *dinF* and *mutT* derivatives. Strains harboring either the empty vector pCA24N (black) or the *dinF*-containing plasmid pDinF (gray) treated with DHR. Data represent the mean values of three independent measurements of the spontaneous fluorescence of 15,000 cells as measured by flow cytometry. The error bars indicate the standard deviation of three independent replicates.
doi:10.1371/journal.pone.0034791.g003

H_2O_2 pre-treatment, as indicated in the experimental procedures section, the expression of *dinF* in the multicopy plasmid pDinF produced a clear decrease in the amount of carbonylated proteins in wild type, Δ*dinF* and Δ*mutT* strains (Fig. 4).

Effect of *dinF* expression on mutation rate

Lac reversion assays were carried out with the strain NR10831 (F′CC101) and its mutant derivatives. The lac assay detects the very specific mutational spectrum of the *mutT* allele (AT to CG) changes in the F′CC101 episome, produced by increased levels 8-oxodGTP in the cell. To further confirm the mutation rates with a different marker, mutation rates to rifampicin resistance were performed. Because NR10831 (F′CC101) is resistant to rifampicin, BW25113 and its mutant derivatives were used. Figure 5 shows that plasmid expression of *dinF* decreased significantly (two tailed Student's t-test; $p<0.05$ in both cases) mutation rate in the Δ*mutT* strains. The decrease was moderate (ten and six-fold for lactose reversion and rifampicin resistance, respectively), suggesting that expression of *dinF* can not cope with the high number of 8-oxodGTP molecules generated in the *mutT* background.

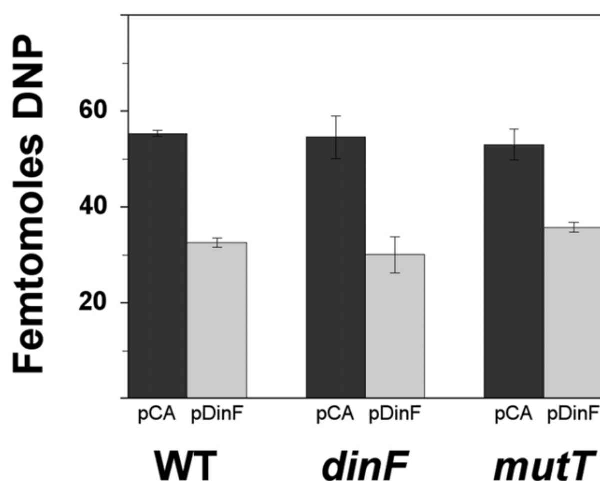

Figure 4. Protein carbonylation. Bar graph quantitating the protein carbonylation (femtomoles of DNP) in cells harboring the empty vector pCA24N (black) or the *dinF*-containing plasmid pDinF (gray) in the wild type, Δ*dinF* and Δ*mutT* derivative strains, following treatment with 10 mM H_2O_2 for 15 min. The data are the mean values from four separate experiments and error bars represent the standard deviation.
doi:10.1371/journal.pone.0034791.g004

lexA-dinF operon in other species

In *E. coli* the *dinF* gene is located a few base pairs after *lexA* (the master repressor of the SOS system) and seems to form a transcriptional unit with it [5], thus suggesting an strict control of *dinF* transcription by the LexA repressor and, consequently an important role for *dinF* in alleviating DNA-damage. In order to investigate whether this co-regulation is maintained in other species, we used operonDB database, which analyzes the co-occurrence of homologous genes together in the same direction and strand in different bacterial sequenced genomes [25]. We found that the *lexA-dinF* operon is maintained in 77 bacterial genomes from those stored in operonDB, including gammproteo-

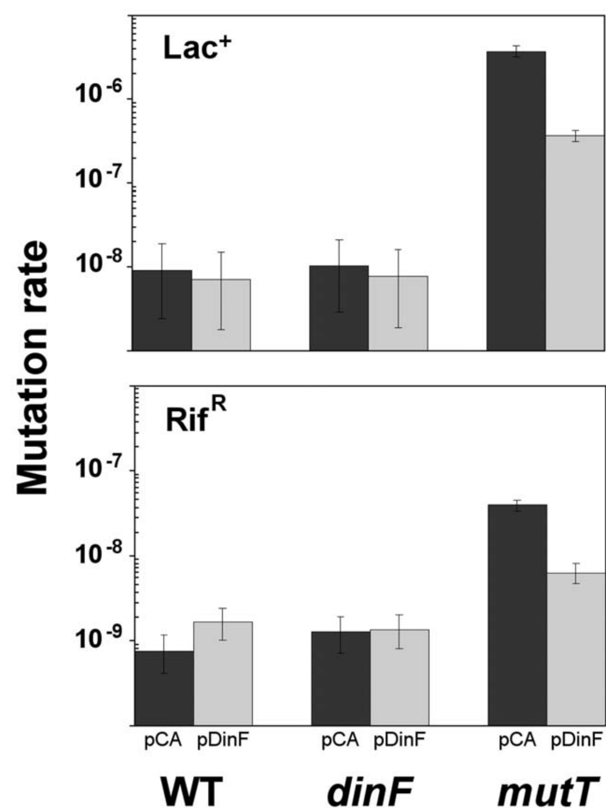

Figure 5. Effect of *dinF* expression on spontaneous mutation rate. The upper graph shows the mutation rate (expressed as mutations/cell/generation) of the Lac^- to Lac^+ reversion for the wild type NR10831 (F′CC101) and its mutant derivatives, Δ*dinF* and Δ*mutT* harboring either the empty vector pCA24N (black) or the plasmid expressing *dinF*, pDinF (gray). The lower plot shows the mutation rate to rifampicin resistance of the wild type BW25113 and its mutant derivatives Δ*dinF* and Δ*mutT* harboring the same plasmids. Values were calculated by the MMS-ML method. Error bars represent 95% confidence intervals.
doi:10.1371/journal.pone.0034791.g005

Table 1. MIC Results.

Product	WT pCA24N 50 μM IPTG	WT pDinF 50 μM IPTG
Ampicillin	16[a]	16
Ceftazidime	0.125	0.125
Streptomycin	8	8
Kanamycin	8	8
Gentamicin	1	1
Ciprofloxacin	0.0625	0.0625
Norfloxacin	1	1
Ofloxacin	0.5	0.5
Bile Salts	**6.25%**	**12.5%**
H_2O_2	1 mM	1 mM
Mitomycin C	4	4
Ethidium Bromide	62.5	62.5

[a]Unless otherwise specified concentrations are expressed in μg/ml.
Bold: Differences found only for this compound.
doi:10.1371/journal.pone.0034791.t001

bacteria from the *Enterobacteriaceae* family (*Citrobacter, Salmonella, Klebsiella, Escherichia* and *Shigella*) and from the *Vibrionaceae* family (*Vibrio cholera, V. fischeri, V. harveyi, V. parahaemoliticus* and *V. splendidus*). Thus, the particular gene order of the *lexA-dinF* operon is maintained in bacteria living in the gastrointestinal tract.

Effect of *dinF* on protection from bile salts killing

Because both *lexA* and *dinF* genes appear to form a single operon only in *Enterobacteria*, we have analyzed whether *dinF* protects from bile salts, a known oxidant product present in the gastrointestinal tract [9]. Table 1 shows that expression of *dinF* in the wild type strain slightly increases MIC of bile salts. To further verify this effect, we performed competition assays between the strains NR10831 Δ*dinF* (pCA24N) and its parental wild type NR10831 (pCA24N) in concentrations of bile salts ranging from 0% to 4%. Figure 6 shows that in the presence of 2% and 4% bile salts, the absence of *dinF* implied a fitness cost of 20% and 50%, respectively (black bars). In order to assess the effect of the complementation of the mutant strain with the plasmid expressing *dinF*, competitions between strains NR10831 Δ*dinF* (pDinF) and wild type NR10831 (pCA24N) were performed. In the same figure 6, grey bars represent the results from these competitions. When pDinF was used to complement the *dinF* deletion, the mutant strain recovered a fitness value similar to that of the wild type strain, having a significant higher fitness (two tailed Student's t-test, $p<0.05$ in both cases). Thus, DinF contributes to final fitness when cells grow in presence of bile salts.

Effect of *dinF* expression on toxic compounds protection

The proteins belonging to the same MATE family, have been described as multidrug efflux pumps able to confer resistance to several antimicrobial agents, such as norfloxacin, ethidium bromide (EtBr), and some aminoglycosides, via a mechanism requiring the proton motive force [26]. In addition, the expression of *dinF* from *Ralstonia solanacearum* in *E. coli* conferred resistance to several toxics, including ampicillin, acriflavine and ethidium bromide [27]. Consequently, expression of the *E. coli dinF* gene in *E. coli* may also confer resistance to some drugs. MICs of ciprofloxacin, norfloxacin, ofloxacin, ampicillin, ceftazidime, streptomycin, kanamycin, gentamycin, bile salts, H_2O_2, mitomycin C and ethidium bromide were determined for the strain Δ*dinF* harbouring either the empty vector pCA24N or the plasmid pDinF. In contrast with what happens with NorM from *E. coli* [28] and DinF from *R. solanacearum* [27], our results indicate that expression of the *E. coli dinF* gene in *E. coli* conferred only a slight resistance to bile salts (Table 1). These results suggest that DinF has a narrower substrate spectrum than other multi-drug resistant efflux pumps of the MATE family.

Discussion

The SOS system is a network that regulates the expression of at least 40 genes, many of them playing key roles in DNA damage tolerance mechanisms, in response to DNA damage [3]. Although the function of most SOS genes is known in *E. coli* and other bacteria [1], there are neither assigned functions nor phenotypes for some others, such as *dinF.* Since it forms a unique transcriptional unit with *lexA,* it would be expected that transcription of *dinF* is tightly regulated by LexA [29]. Consequently, any agent with the potential to induce transcription of *lexA* would, in principle, induce that of *dinF.* Apart from the classic SOS inducers, including UV, mitomycin C and gamma-radiation, increased levels of ROS have been reported to induce the SOS response [30,31]. To protect all cellular components, including DNA, proteins and lipids, from damage, rapid and coordinated responses are essential for all living organisms. Natural selection has produced a number of systems to prevent or repair DNA damage. Post-replication mismatch repair system (MMR) mainly repairs replication errors [32]. Endogenous DNA damage is primarily repaired by base excision repair (BER) (for a review see reference [1]). Very important are oxidative DNA lesions, which play a major role in spontaneous mutagenesis [33]. Oxidation of guanine to 7,8-dihydro-8-oxoguanine (8-oxoG) is especially noteworthy because, if not repaired, this base lesion can be bypassed by DNA polymerases and originate mutations [34,35]. In *E. coli* there are specialized proteins, belonging to the so-called GO system, dedicated to alleviate the mutagenicity of 8-oxodGTP [22]. One of these proteins is the MutT enzyme, a nucleoside

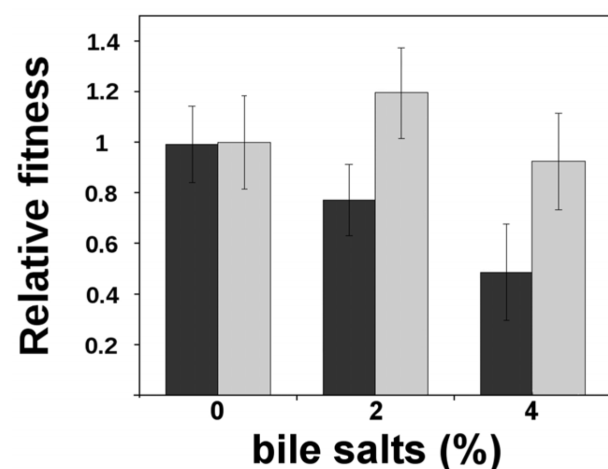

Figure 6. Fitness under bile salts challenge. Black bars represent the mean results of competition experiments between the NR10831 Δ*dinF ara*[+] (pCA24N) and its parental wild type strain NR10831 *ara*[−] (pCA24N), and viceversa. Grey bars represent the mean results of competition experiment of strain NR10831Δ*dinF* (pDinF) *vs* NR10831 (pCA24N) in order to see the effect of the complemented mutant. Error bars represent 95% confidence intervals around the mean.
doi:10.1371/journal.pone.0034791.g006

triphosphate pyrophosphohydrolase, which converts 8-oxodGTP to 8-oxodGMP and pyrophosphate, and inactivates this mutagenic activity. In the absence of MutT there is an increase in AT to CG mutations [22]. Interestingly, *E. coli* and *Pseudomonas aeruginosa mutT*-deficient strains are severely impaired in survival under hydrogen peroxide challenge [36]. MutT also hydrolyzes 8-oxoGTP, preventing incorporation of 8-oxo-Gua into RNA [37]. Recently, NorM, a MATE-family efflux pump, has been demonstrated to protect the cell from the increased hydrogen peroxide killing caused by the lack of MutT [8].

Multi-drug efflux pumps, able to extrude chemicals that can potentially damage DNA, RNA, and proteins, have appeared and been refined through evolution [38]. Therefore, one might speculate that some of these proteins may have also evolved a transcriptional regulation related with the SOS system. However, the expression of none of these proteins is known to be regulated by LexA [3]. According to its deduced polypeptide sequence, DinF is the prototype of a branch of the new family of multidrug and toxin compound extrusion (MATE) membrane proteins [34,35]. Thus, it is tempting to speculate that DinF could be the first described multidrug efflux pump whose transcription is regulated in response to DNA-damage. In this work we show that expression of DinF is able to reduce the level of intracellular ROS leading, putatively, to the prevention of protein oxidative damage, mutagenesis and the protection from peroxide killing. Despite of the fact that the efflux activity of the *E. coli* DinF protein has still to be proved, our results suggest that DinF may reduce the intracellular pools of potentially oxidizing molecules, diminishing the level of 8-oxodGTP, as suggested by the reduction of the mutation rate of the very specific spectrum of mutations (AT to CG) produced in the *mutT* background.

Cellular extrusion systems in bacteria may protect against toxic compounds like antibiotics and biocides [39], nevertheless, we are far from understanding the real functions due to failing in the identification of its natural substrates. Despite the data obtained with NorM from different bacterial species [15,26] and DinF from *R. solanacearum* [27], our results suggest that DinF from *E. coli* is not involved in the resistance to antibiotics and other toxics, except bile salts.

Our data from competitions between wild type and the *dinF* mutant, together with data from competitions between wild type and the complemented mutant, clearly show that *dinF* is involved in protection against bile salts. Moreover, the effect of *dinF* expression on H_2O_2 viability, intracellular ROS levels, protein carbonylation and mutation rate in the *mutT* background strongly suggest that bile salt protection can be exerted *via* the reduction of oxidative damage. Finally, the putative strict control of *dinF* transcription by LexA, the master repressor of the SOS system, only in bacteria facing bile salts insults, suggests that this association (*lexA-dinF* operon) has evolved to protect these bacteria against this kind of host defenses. This hypothesis is consistent with the fact that exposure of *Salmonella enterica* to bile salts induces the SOS response, indicating the DNA-damaging activity of bile salts [9].

In summary, we describe here for the first time a role for the SOS-gene *dinF*: protection of DNA and proteins from oxidative molecules and reduction of mutation rate when MutT activity is absent (i.e. increased levels of 8-oxo-dGTP). An especially interesting case of bile/pathogen interaction is found in *S. enterica*, which is exposed to bile in the lumen of the mammalian intestine, where concentrations of bile salts range from 0.2 to 2% [40], and in the gall bladder, where much higher concentrations of bile are found [41]. In addition, one of the main pathogens causing cholecystitis is *E. coli* [41,42].

According to the predicted function of DinF and its ability to reduce the intracellular ROS levels, it is tempting to speculate that the DinF protective activity could be exerted *via* the extrusion of oxidizing molecules. Since hydrogen peroxide diffuses rapidly through membranes, it seems unlikely that DinF might relieve H_2O_2 stress by pumping H_2O_2 out of the cell. The presence of the *lexA-dinF* operon only in species from the *Enterobacteriaceae* family, together with the bile salts protection, suggests a bile protective role for DinF in this bacterial family. At this stage of the investigation, the exact nature of the DinF activity remains unknown and requires further studies. Because DinF homologues have been found in all three domains of life, including humans [34,35], it is conceivable that some of them are also involved in ROS protection.

Acknowledgments

We thank the National Institute of Genetics of Japan and I. Fijalkowska from the Institute of Biochemistry and Biophysics of Warsaw for kindly providing some of the strains.

Author Contributions

Conceived and designed the experiments: JB JR AR JRG AC. Performed the experiments: JB JR AR JRG AC. Analyzed the data: JB JR AR JRG AC. Contributed reagents/materials/analysis tools: JB. Wrote the paper: JB JR AR JRG AC.

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3. Alta frecuencia de recombinación en cepas extra-intestinales de *Escherichia coli*

El objetivo que perseguimos en el artículo presentado a continuación[3] fue el de esclarecer cuál es el potencial evolutivo de *E. coli*, utilizando para ello una colección de 160 cepas naturales. Como se ha descrito en la introducción, la evolución bacteriana está esencialmente (pero no únicamente) basada en mutación y recombinación. Conocer la magnitud de estos parámetros es de vital importancia para entender cual es la contribución potencial de cada una de estas fuerzas en el desarrollo de resistencia a antibióticos o para comprender la transición entre comensalismo y patogenicidad.

Dado que en la literatura existen numerosos estudios enfocados a la cuantificación de la mutagénesis espontánea (15, 16, 21, 158), decidimos volcar nuestro interés en elucidar cual es la variabilidad en poblaciones naturales con respecto la recombinación homóloga. La mayoría de estudios poblacionales precedentes habían usado métodos indirectos (basados en la huella genómica que deja cada evento de recombinación) para inferir la contribución de la recombinación en el modelado del genoma, ofreciendo una "foto" estática de la historia evolutiva de cada cepa. El objetivo directriz del trabajo que se presenta a continuación fue el de complementar esa visión con una medida directa del potencial para recombinar secuencias homólogas en *E. coli*, usando herramientas genéticas que nos permitieron realizar estimaciones de la tasa de recombinación intrínseca. Para ello construimos un sistema basado en un plásmido y lo introdujimos en una colección de cepas bien caracterizada (21, 158, 159).

El primer resultado es que la recombinación es enormemente variable, ya que encontramos cepas con frecuencias que se extienden a lo largo de varios órdenes de magnitud. Además es dinámica y, como la mutación, sufre variaciones según el ambiente, hecho que se manifiesta particularmente cuando se mide en orina humana, un

3. Cuyo material suplementario puede consultarse en el anexo III

medio que habitualmente forma parte del hábitat de patógenos extra-intestinales de *E. coli*.

Pero el resultado más sorprendente se obtuvo al clasificar cada cepa según su relación trófica con el hospedador humano del que fueron aisladas: Los patógenos extra-intestinales de *E. coli* presentan mayores frecuencias de recombinación que sus congéneres comensales. Asimismo, encontramos una asociación positiva entre la frecuencia de recombinación y el número de genes de virulencia presentes en cada cepa, lo que refuerza la idea de que la virulencia y la recombinación están estrechamente relacionadas.

Los resultados presentados aquí contribuyen al desarrollo del conocimiento sobre los determinantes que caracterizan la evolución de las bacterias patógenas y cuáles son las principales diferencias con sus congéneres comensales, lo que sugiere que el camino adapativo

High Recombinant Frequency in Extraintestinal Pathogenic *Escherichia coli* Strains

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Associate editor: Csaba Pal

Abstract

Homologous recombination promotes genetic diversity by facilitating the integration of foreign DNA and intrachromosomal gene shuffling. It has been hypothesized that if recombination is variable among strains, selection should favor higher recombination rates among pathogens, as they face additional selection pressures from host defenses. To test this hypothesis we have developed a plasmid-based method for estimating the rate of recombination independently of other factors such as DNA transfer, selective processes, and mutational interference. Our results with 160 human commensal and extraintestinal pathogenic *Escherichia coli* (ExPEC) isolates show that the recombinant frequencies are extremely diverse (ranging 9 orders of magnitude) and plastic (they are profoundly affected by growth in urine, a condition commonly encountered by ExPEC). We find that the frequency of recombination is biased by strain lifestyle, as ExPEC isolates display strikingly higher recombination rates than their commensal counterparts. Furthermore, the presence of virulence factors is positively associated with higher recombination frequencies. These results suggest selection for high homologous recombination capacity, which may result in a higher evolvability for pathogens compared with commensals.

Key words: **homologous recombination, evolution, ExPEC, commensal, RecA.**

Introduction

Two types of genetic mechanisms allow bacterial genomes to diversify and therefore adapt to new or changing environments. On the one hand, inherent errors in the replication processes through point mutations, insertion sequence mobility, or genomic rearrangements lead to the transmission of modified genomes from one generation to the next. On the other hand, import of exogenous DNA, a process termed horizontal gene transfer (HGT), may lead to the immediate acquisition of either new functions or new alleles of existing genes through homologous recombination (HR). When the DNA sequences acquired by HGT are unable to replicate autonomously, HR can contribute both to its integration in the bacterial chromosome and to its propagation in the species using the conserved regions flanking the integration site (Schubert et al. 2009).

Therefore, recombination, which is a major DNA repair pathway, is also a key factor in bacterial genomic evolution. Through HGT, HR allows bacteria to expand their energy sources, evade the immune response of their hosts, acquire antibiotic resistance, and increase virulence (Guttman and Dykhuizen 1994; Lawrence and Roth 1996; Lawrence and Ochman 1998). Moreover, in *Escherichia coli* species it has been estimated that recombination is much more frequent than mutation (Touchon et al. 2009). For instance, a nucleotide has a 50–100 times higher probability of being involved in genetic recombination than being mutated (Guttman and Dykhuizen 1994). Computer simulations and experimental results suggest that high recombination rates may combine beneficial mutations appearing in different backgrounds faster than the incremental accumulation of them (Tenaillon et al. 2000; Cooper 2007). Recombination, therefore, can accelerate the rate at which a bacterial population adapts to environmental conditions (Levin and Cornejo 2009).

Obviously, bacteria that share ecological niches have more opportunities for genetic exchange than those living in distinct environments. The acquisition and use of premade and pretested new traits, such as antibiotic-inactivating enzymes or virulence mechanisms, provide a broad spectrum of possibilities to bacteria, which may use new functions previously developed by others. In environments with a high possibility for genetic exchange (where many related and/or genetically compatible species are present in the niche), the genetic exchange, through plasmids, transposons, phages

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and/or HR, may be favored over mutation (Tenaillon et al. 2000). On the contrary, genetically isolated bacterial populations (i.e., with low possibility of exchanging DNA) have to find their own solutions through their own resources (i.e., mutation and/or gene shuffling by HR) and, consequently, will benefit from increased mutation and intragenomic recombination rates. A paradigmatic example is *Mycobacterium tuberculosis*, in which, for instance, acquisition of antibiotic resistance occurs exclusively by mutation (Ramaswamy and Musser 1998). Thus, the rates and effects of mutation and recombination on evolution are drastically conditioned by ecology (Didelot and Maiden 2010).

Both mutation and recombination processes are genetically controlled. As such, they are themselves under the action of natural selection. Both processes can therefore evolve within species through a process called second order selection (Tenaillon et al. 1999) in which variants are selected for based on their impact on genomic diversity. For instance, a high mutation rate clone, or mutator, can be selected for (Sniegowski et al. 1997; Oliver et al. 2000) or counterselected (Trobner and Piechocki 1984; Wielgoss et al. 2013) based on the selective impact of the mutation it generates (Tenaillon et al. 1999). Mutators are favored by selection when the advantage of beneficial mutations is greater than the cost of being a mutator due to the overproduction of lethal and deleterious mutations (Mao et al. 1997; Taddei et al. 1997). Mutator alleles have been extensively studied in *E. coli* where a large diversity of mutation rates can be found among natural isolates (LeClerc et al. 1996; Matic et al. 1997; Denamur et al. 2002; Denamur and Matic 2006). In most cases, bacterial mutator strains had a mismatch repair deficient genotype (LeClerc et al. 1996; Matic et al. 1997; Oliver et al. 2000, 2002). However, much less is known about the diversity of recombination rates within a bacterial species. Yet, for a species like *E. coli* living in the promiscuous gut of vertebrates (containing large numbers of cells, species and niches that facilitate genetic exchange) (Stecher et al. 2013) and having a highly plastic genome, it can be expected that recombination should be a major player of adaptation.

Escherichia coli is a versatile and ubiquitous bacterial species that colonizes the gut of vertebrates and is also a leading human pathogen, causing chronic and acute infections, the management of which can be severely complicated by antibiotic resistance. This apparent dichotomy in bacterial lifestyle has been linked to the acquisition of particular gene clusters, called pathogenicity islands (PAIs), which in great part confer *E. coli* its pathogenic capacity (Groisman and Ochman 1996; Hacker and Kaper 2000). However, according to the coincidental-evolution hypothesis (Le Gall et al. 2007; Diard et al. 2010), virulence determinants, at least for extraintestinal pathogenic *E. coli* (ExPEC), can be intestinal colonization and survival factors, that is, acquisition of virulence could be a byproduct of commensalism. Independently of the forces selecting for virulence, *E. coli* is a perfect candidate for studying the transition between commensalism and pathogenicity.

The extant of recombinational diversity and its potential link with virulence have been studied before with genomic approaches. Data from multilocus sequence typing (MLST) indicate that rates of evolution were accelerated in *E. coli* pathogenic strains by increased HR events, suggesting that recombination and virulence are causally related (Wirth et al. 2006). Although genomic techniques, such as MLST and whole-genome sequencing, are successfully being used to understand how mutation and recombination impact bacterial evolution (Tenaillon et al. 2000; Spratt et al. 2001; Wirth et al. 2006; Touchon et al. 2009; Didelot et al. 2012), some important caveats have not been addressed. First, genetic events cannot be disentangled from the selection process and the DNA transfer process itself; consequently, only the signature of their combined action can be analyzed (Denamur et al. 2010). Second, MLST relies on the sequences of a relatively small number of house-keeping genes (usually seven) and therefore it is prone to introduce misleading results because it overestimates the weight of single gene fragments, which provide little information on the overall genomic composition (Falush et al. 2006). Furthermore, it has been pointed out that it lacks the resolution necessary for ascertaining the underlying diversity in bacterial populations determined by other approaches (Noller et al. 2003). Third, it is plausible that during a period of stress, enhanced recombination or mutation rates could be transitorily selected (Bjedov et al. 2003). These episodic increased rates of adaptability are difficult to distinguish with these techniques, as they are based on final nucleotide differences and hence lack temporal resolution (Paul et al. 2013).

We therefore decided to analyze the intrinsic capacity of recombining homologous DNA sequences, independently of the DNA transfer, selective processes and mutational interferences, in a collection of well-characterized human *E. coli* natural isolates from different origins, including both commensals and extraintestinal pathogens (Picard et al. 1999; Duriez et al. 2001; Hommais et al. 2005). Correlations between recombinant and mutant frequencies, isolate origin, phylogenetic group, number of virulence genes, and pathogenic capacity have been studied.

Results

Spontaneous Mutagenesis

Spontaneous mutagenesis of the strains was estimated by monitoring their capacity to generate mutations conferring resistance to rifampicin (RifR). Figure 1*A* shows the distribution of RifR mutant frequencies of both commensals and pathogens (raw data are shown in supplementary table S1, Supplementary Material online). As previously described (LeClerc et al. 1996; Matic et al. 1997; Denamur et al. 2002), mutant frequencies of most strains fall into a narrow peak (around 10^{-8}), except for a small group of strains, defined as mutators. Median values of both populations were almost identical (9.3×10^{-9} and 1.5×10^{-8}, respectively) (fig. 1*B*). The proportion of mutators is about 2.3-fold higher in the group of pathogen strains (10% vs. 4.4%), although this difference was not significant at the 95% level (Fisher's Exact Test $P = 0.21$). In our study, strains were considered mutators when they exhibited mutant frequencies of at

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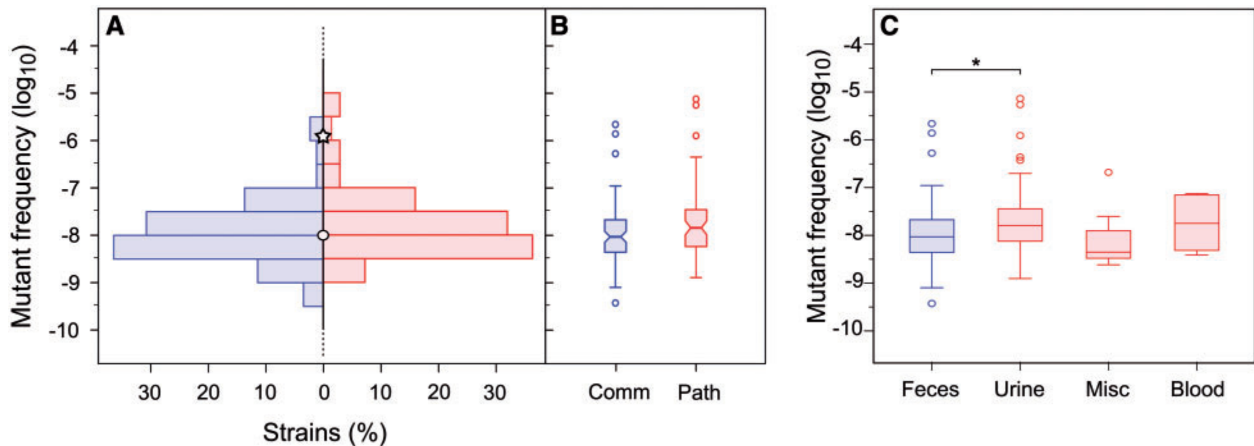

FIG. 1. RifR mutant frequencies. (*A*) Histogram showing the distributions of mutant frequencies of 90 commensals (left) and 70 pathogens (right). Frequencies of the *Escherichia coli* K-12 strains ME12 (circle) and ME12 Δ*mutS* (star) are shown as a reference. (*B*) Boxplot representing median values (horizontal line in the box) of mutant frequencies of commensals and pathogens. (*C*) Distributions of mutant frequencies according to the origin of the isolates. The differences between mutant frequencies of fecal and urinary strains are statistically significant (pairwise Wilcoxon test $P = 0.005$). For all boxplots, the depth of the box represents the interquartile range (50% of the data) and the whiskers extend to 1.5 times the interquartile range. Notches around medians represent 95% confidence intervals.

least 10-fold higher than the median value of all studied strains ($n = 160$; median = 1.06×10^{-8}).

As previously reported (Denamur et al. 2002), differences in the distribution of mutant frequencies can be observed between commensal ($n = 90$) and uropathogenic ($n = 48$) strains (pairwise Wilcoxon test $P = 0.005$) (fig. 1*C*).

Homologous Recombination

To study HR, we developed a genetic assay (a detailed description of the process can be found in the Supplementary Material online), which scores the recombination between two truncated *tetA* alleles separated by an antibiotic resistance cassette. Recombination restores the functional *tetA* gene, thereby conferring tetracycline resistance, which can be selected for. Therefore, this assay allows quantification of the frequency of recombinants.

We tested that our system relied on HR by introducing the plasmid pRhomo, conferring resistance to ampicillin and kanamycin but not to tetracycline (AmpRKanRTetS) (supplementary fig. S1, Supplementary Material online), carrying the recombination testing system, into the *E. coli* K-12 MG1655 derivatives ME12 and ME12 *recA* (Elez et al. 2007). When a recombinant event occurs between the two *tetA* alleles, plasmid pRhomo-Tet (TetR) is generated (supplementary fig. S1, Supplementary Material online). The frequency of AmpRTetR recombinants was in the order of 10^{-3} for the wild-type strain ME12, a value similar to that obtained with other tests (Elez et al. 2007; Lopez et al. 2007). As expected, HR was about 100-fold lower in the strain lacking *recA* (see values for K-12 in supplementary fig. S2, Supplementary Material online), indicating that reconstruction of the functional *tetA* is mostly dependent on RecA, and that it therefore relies on HR. The *recA* requirement was also studied in 13 randomly chosen strains from our panel (five commensals and eight pathogens). These strains, made *recA*-deficient (*recA*::*kan*) as explained in Supplementary Material online, showed a decreased rate of HR with respect to their *recA*-proficient counterparts in all cases (supplementary fig. S2 and table S1, Supplementary Material online). For all tested strains, the RecA-dependent recombination is higher than 95% (with many strains in which the dependence is over 99.9%). Exceptions are strains P53 and P70 (with a RecA-dependence of 72% and 83%, respectively). Thus, in our conditions, the RecA-independent recombination seems to be rare in most cases. DNA-polymerase slippage and/or RecA-independent DNA crossing over events (Lovett et al. 1993) may account for the observed RecA-independent recombination. Furthermore, a strong dependence on the RecA function has been described previously in assays where DNA is acquired from other cells through F′ transfer (see e.g., Matic et al. 1995). Therefore, the use of plasmid pRhomo to study the frequency of *recA*-dependent HR was validated.

In addition, to ascertain that plasmidic recombination measured with pRhomo is comparable to chromosomal recombination, we introduced a single copy of a DNA fragment containing two pieces of the *tetA* gene separated by a kanamycin resistance gene (*tetA1-kan-tetA2*) into the chromosome of MG1655 K-12 and its *recA*-isogenic mutant, generating the strains MG1655 *att*Tn7::*tetA1-kan-tetA2* and MG1655 *att*Tn7::*tetA1-kan-tetA2 recA* (for a detailed description, see supplementary material and fig. S3, Supplementary Material online). The chromosomal recombination events in the *recA*-deficient strain were almost ten times less frequent (3.2×10^{-6}) than in wild-type MG1655 (1.63×10^{-5}) (supplementary fig. S4, Supplementary Material online). This confirms that, whether on a plasmid or inserted as a single copy in the chromosome, the *tetA* fragments mostly undergo *recA*-dependent recombination. Note that when *tetA* sequences are in the chromosome, the frequency of TetR recombinants is lower than that of the plasmidic construction (10^{-5} vs. 10^{-3}), indicating that, as expected, the availability of substrates for recombination influences the frequency of recombinants. To further explore this in our strains, we measured the recombinant frequencies of five

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strains of our collection whose genomes have been sequenced (*E. coli* B, F11, HS, 536, and CFT073) (supplementary table S1, Supplementary Material online) and the *E. coli* MG1655 derivative ME12 strain harboring either the plasmidic or the chromosomal system. A strong linear correlation (Pearson's $r = 0.82$, $P = 0.04$) between recombinant frequencies obtained with both systems was observed (supplementary fig. S5, Supplementary Material online), demonstrating that pRhomo is an accurate tool to measure recombinant frequency. It should be noted that our assay with pRhomo cannot distinguish between intra- and interplasmidic recombination. However, our data indicate that if both possibilities occur, the recombinant frequencies still correlate with those from the chromosomal assay and are mainly mediated by RecA.

Recombinant frequencies of three independent colonies of each of the 160 strains (90 commensal and 70 pathogens), containing plasmid pRhomo, were calculated as the median number of recombinants (AmpRTetR) divided by the median number of viable cells on Lysogeny Broth (LB)-agar plates containing only ampicillin. Figure 2 shows the histogram (*A*) and the boxplot (*B*) with the distribution of recombinant frequencies of commensal and pathogen isolates. Interestingly, a high variability of recombinant frequencies, ranging from 10^{-1} to 10^{-9}, is observed for both groups. Our results clearly demonstrate that altogether pathogens have higher recombinant frequencies than commensals (Mann–Whitney *U* test $P < 10^{-8}$), with medians of 6.46×10^{-4} and 9.94×10^{-6}, respectively. An analysis of frequencies, classifying the strains by the isolate origin, showed significant differences (pairwise Wilcoxon test $P < 0.003$, in all cases) when comparing commensal strains with pathogenic strains from urine and also with the miscellaneous group, which includes isolates from diverse extraintestinal infections (mainly from pus) but excluding urine and blood (fig. 2*C*). Interestingly, recombination of pathogens isolated from blood showed no statistical differences with that of commensal strains (fig. 2*C*).

Estimation of recombinant frequencies by using pRhomo could depend on the different copy number of the plasmid in each strain (in principle, the higher plasmid copy number the higher probability of producing a recombination event). To rule out this possibility, the number of copies of pRhomo was determined for a group of strains randomly chosen ($n = 28$, 14 commensals and 14 ExPEC), with different frequencies of recombinants. The K-12 ME12 strain, which showed approximately 500 copies of pRhomo per cell (see supplementary methods, Supplementary Material online), was used as a reference. No correlation between recombinant frequency and plasmid copy number was observed ($n = 29$, Spearman's rho = 0.13, $P = 0.45$) (supplementary fig. S6, Supplementary Material online), indicating that, although the availability of substrates for recombination can influence the frequency of recombinants (see above), the observed differences in recombinant frequencies cannot be explained by the differences in the number of plasmid molecules present in each strain.

Escherichia coli constantly alternates between various habitats. In particular, ExPEC are characterized by the presence of virulence factors, which allow surviving and growing in host niches with specialized defenses, where they are responsible for severe infections. The most common site of infection for ExPEC is the urinary tract. For this reason and because the highest difference in recombinant frequency was observed between commensals and urinary tract infection strains (fig. 2*C*), we wanted to compare frequencies measured in LB and human urine. Figure 3 shows the recombinant frequencies of 22 strains (13 ExpEC and 9 commensal)

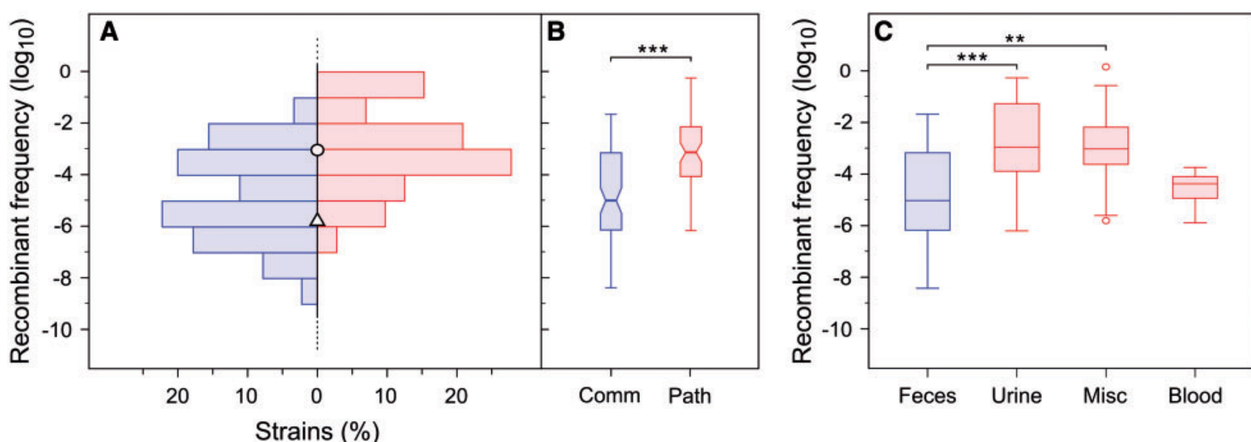

Fig. 2. TetR recombinant frequencies. (*A*) Histogram with the distributions of TetR recombinant frequencies of 90 commensal (left) and 70 pathogen (right) isolates. Frequencies of the *Escherichia coli* K-12 strains ME12 (circle) and ME12 Δ*recA* (triangle) are shown as a reference. (*B*) Boxplots of the distributions of recombinant frequencies of commensals and pathogens. Stars indicate a highly significant difference between distributions according to the Mann–Whitney *U* test (*P* value $< 10^{-8}$). (*C*) Boxplots representing the median values for strains grouped by origin of the isolates. Significant differences were found between strains isolated from the feces of healthy individuals and those isolated from urine and miscellaneous infections (Wilcoxon pairwise test; $**P < 0.003$, $***P < 10^{-5}$). For all boxplots, the horizontal line represents the median value, the depth of the box represents the interquartile range (50% of the data), and the whiskers extend to 1.5 times the interquartile range. Notches around medians represent 95% confidence intervals.

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harboring pRhomo measured in LB and urine. Our results indicate that growth in urine dramatically modifies recombinant frequency. Although most strains (18 of 22; 14 with differences statistically significant) increased their recombinant frequencies (from 4- to 10,000-fold) upon growing in urine, the increase is not a general phenomenon (three strains showed decreased frequency and one strain showed no difference between LB and urine). This, however, confirms that environment may condition the rate of recombination, adding a level of complexity to the generation of variability. Increased recombination in urine was also observed in the strains MG1655 *att*Tn*7*::*tetA1-kan-tetA2* and MG1655 *att*Tn*7*::*tetA1-kan-tetA2 recA* (supplementary fig. S4, Supplementary Material online). The difference in the recombinant frequency in urine between these two strains indicates that recombination is mainly *recA*-dependent in this environment. To explore the generality of this *recA*-dependence in urine, the recombinant frequencies of the 13 *recA*-deficient strains (five commensals and eight pathogens) from our panel were studied in this medium. In all cases except one (12 of 13), the *recA*-deficient strains showed lower recombination frequencies in urine than their *recA*-proficient counterparts (supplementary fig. S7, Supplementary Material online).

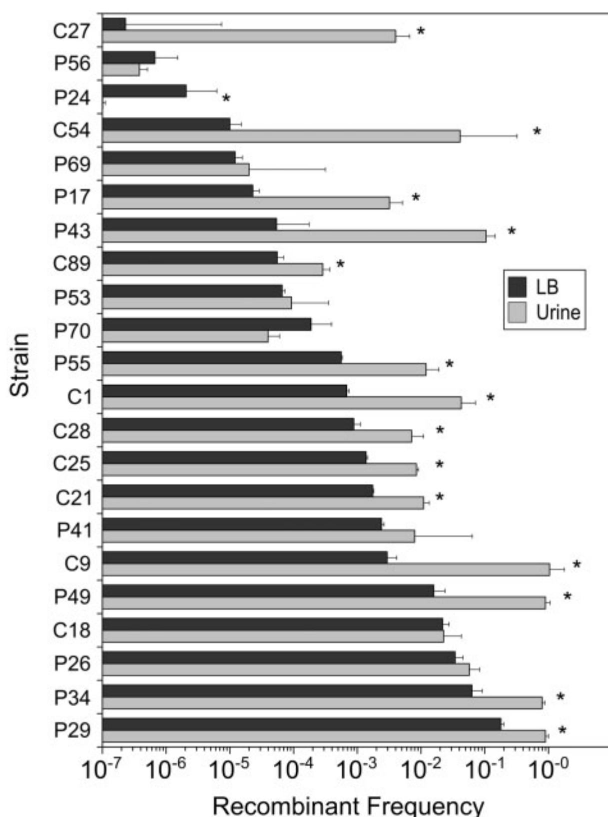

Fig. 3. TetR recombinant frequencies in LB and urine. Frequencies (median ± SEM) measured in a subset of 22 pRhomo transformed *Escherichia coli* strains in LB (black bars) and urine (gray bars) are shown. Strain names begin with either "P" or "C" indicating that the strain is a pathogen or a commensal, respectively. The asterisk denotes significant differences (*t*-test for log10-transformed values; $P < 0.05$) between the frequency in urine and in LB. To facilitate understanding, strains appear ordered by recombinant frequency in LB.

Recombinant Frequency and Number of Virulence Genes

The presence of different extraintestinal virulence genes was previously studied in 116 of our strains (Picard et al. 1999; Duriez et al. 2001). Therefore, we analyzed the correlation of recombinant frequencies and the number of virulence genes of 116 strains (71 commensals and 45 pathogens). Taking into account that pathogens have higher recombinant frequencies than commensals and also a higher number of virulence genes, a correlation between recombinant frequency and the number of virulence genes was suspected. Our results demonstrate that there is a modest, yet significant, positive correlation ($n = 116$, Spearman's rho = 0.23, $P = 0.0127$) between recombinant frequency and the number of virulence genes (fig. 4). However, no correlation was observed between recombination and pathogenicity, measured as the intrinsic virulence of the strains estimated by a mouse sepsis assay (Johnson et al. 2006) although, in this case, there are not enough commensal strains with data to perform a statistically significant study.

In addition, no correlation could be established between frequencies of recombinants and mutation, that is, strains with higher recombinant frequency did not have higher mutant frequency, or between mutation and number of virulence genes. Furthermore, no statistically significant differences were found in mutant frequency or in HR among phylogenetic groups (Kruskal–Wallis test; $P > 0.15$).

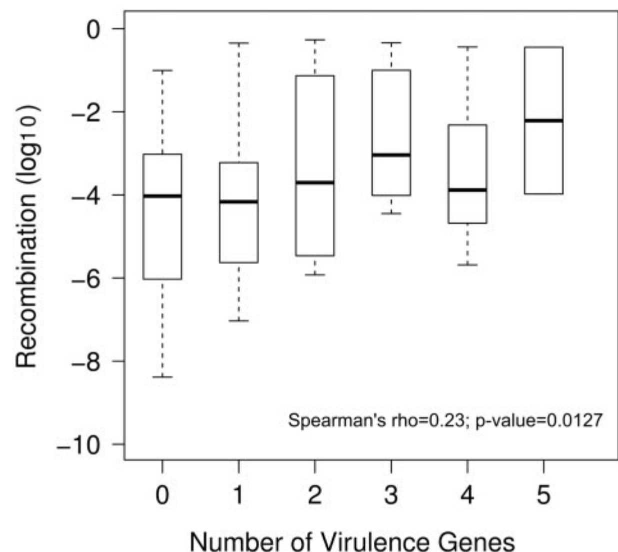

Fig. 4. Recombinant frequency of strains grouped by number of virulence genes. Boxplots represent the median values of recombination frequency of strains grouped by number of virulence genes. A positive correlation (Spearman's rho = 0.23, $P = 0.0127$) can be identified. Strains with more virulence determinants tend to have higher median values of recombination (horizontal line in the boxplot). The depth of the box represents the interquartile range (50% of the data) and the whiskers extend to 1.5 times the interquartile range.

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Discussion

Because the *E. coli* species includes both commensal and pathogenic strains and they are easy to isolate and grow, it has been used for years as a model to study population genetics and the transition between commensalism and pathogenicity. Different techniques, such as serotyping, biotyping, random amplified polymorphic DNA, restriction fragment length polymorphism and multilocus enzyme electrophoresis, were used in the first population genetics studies. These studies suggested a clonal structure of the *E. coli* species, whose evolution was largely dominated by mutation (see e.g., Tenaillon et al. 2010, and references therein). Advances in molecular technology have permitted the refinement of results on mutation and recombination at the molecular level, demonstrating that recombination is a key parameter in the evolution of *E. coli* (Schubert et al. 2009; Tenaillon et al. 2010). However, although these new genetic techniques are very useful for studying the effect of mutation and recombination on bacterial evolution, they present a series of drawbacks, including low resolution and incapacity to separate genetic events from the process of selection.

It is generally admitted that PAIs are not solely vertically transmitted, but spread within the *E. coli* species by recombination. For instance, PAIs can be transferred between different *E. coli* strains by F-plasmid mediated mobilization, demonstrating that HGT and HR play major roles in horizontal transfer of PAIs within the *E. coli* species (Schubert et al. 2009). Wirth et al. (2006) suggested that *E. coli* pathogenic strains have undergone increased rates of recombination in genes distributed throughout the genome, likely accelerating the evolution to pathogenicity. As a consequence, they proposed that recombination and virulence are causally related. However, these results relied on the hypothesis that *E. coli* had four phylogenetic subgroups and clustered all strains not belonging to these groups as recombinants (Falush et al. 2006; Denamur et al. 2010). We now know that there are close to seven solid phylogenetic groups in the species (Clermont et al. 2013). Therefore, the question of the link between recombination and virulence has not been solved yet.

We decided to test more directly whether or not *E. coli* pathogenic strains facing, in principle, higher selection pressures from host defenses than commensals, are more prone to adaptive evolution by HR than their commensal counterparts. Guided by this important evolutionary question, we have analyzed the actual potential for genetic change (mutation and HR) of a panel of well-characterized naturally occurring *E. coli* strains, including both commensals and extraintestinal pathogens, from different origins. We have experimentally characterized both mutant frequency and the rate of recombination between homologous DNA sequences, and studied the relationship of these parameters with the isolate origin, phylogenetic group, number of virulence genes, and pathogenicity. Note here that our experimental approach exclusively permits the study of the process that allows recombination between homologous DNA sequences, which is, obviously, only a part of the whole process of HGT.

Concerning the variability of mutation rate, we corroborate the results found in previous studies (LeClerc et al. 1996; Matic et al. 1997). We show the existence of some variability but no significant differences between mutant frequencies of commensal and ExPEC strains. However, differences in the distribution of mutant frequencies can be observed between commensal and uropathogenic strains, confirming previous results from Denamur et al. (Denamur et al. 2002).

The results for HR are surprisingly much more marked. First, the diversity of recombinant frequency observed extends over many orders of magnitude. Hence the ability to recombine homologous DNA, as measured by our system, is extremely diverse within the species: Although some clones show 10% of recombinants other show one in a billion. Second, these frequencies are significantly enhanced in the environment most commonly encountered by ExPEC, that is, urine, as demonstrated for a group of strains. This means that recombination is variable not only among strains but also among environments. Third and most importantly, our results indicate that ExPEC strains have a significantly higher frequency of recombinants (about 2 orders of magnitude) than commensal isolates. This is also associated with a significant positive correlation between recombinant frequencies with the number of virulence genes of each strain.

These results suggest that, as for mutation rate, which shows both diversity among strains and among environments (Bjedov et al. 2003), selection can act on the rate of recombination within *E. coli*. The large diversity we observed is a prerequisite for selection to be able to operate. Upon arrival in a new environment, the benefits linked to the acquisitions of some foreign genetic material present in other strains, such as PAIs, or the acquisition of different alleles (or its generation by intrachromosomic gene shuffling) promoting adaptation to local conditions, may drive the selection for higher recombination rates. According to that hypothesis, the difference between commensals and pathogens suggests that pathogens may have been facing more challenging environments than their commensal counterparts. Moreover, it suggests that thanks to this enhanced recombination, ExPEC may have an increased potential for further adaptive evolution through recombination. It is important to highlight that, although HR is a key step in the overall HGT process, selection could also act on other steps, such as the rate of DNA transfer.

On the other hand, the intensity of intrinsic oxidative stress appears to be quite variable among natural isolates of *E. coli* grown in urine (Aubron et al. 2012). Because oxidative stress is responsible for DNA damage (Imlay 2013) and recombination is a major DNA repair pathway, increased recombination rates may also be selected by their higher capacity to cope with the stressful environment represented here by human urine. These hypotheses may not be mutually exclusive, as both roles of recombination (DNA-repair and the acquisition of new alleles) can be subjects of selection and act synergistically. However, regardless of the nature of the selective pressure acting on recombination, the resulting increased

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variability may play an important role in bacterial evolution in a manner similar to what has been suggested for stress-induced mutagenesis (Tenaillon et al. 2004).

Overall, our results demonstrate that a large diversity in the rate of HR exists within *E. coli* species and that this diversity may correlate with the life style (commensal or pathogen). Further work will have to be done to unravel the mechanisms and the selective forces driving this recombination diversity and to uncover its dynamics and long-term impact on bacterial species genetic diversity.

Materials and Methods

Bacteria, Plasmids, and Primers

Escherichia coli K-12 strains, plasmids, and primers used in this study are shown in table 1 and supplementary table S2, Supplementary Material online.

Commensal and Pathogenic *E. coli* Strains

In total, 160 human *E. coli* strains (90 commensal strains, isolated from feces of healthy persons, and 70 extraintestinal pathogens involved in various pathologies) from well-characterized collections gathered in the 1980s from different countries (France, Croatia, Germany, Mali, Canada, the Unites States, Australia) (Picard et al. 1999; Duriez et al. 2001; Hommais et al. 2005), were used for this study. Main characteristics and all experimental data utilized in this work are shown in the supplementary table S1, Supplementary Material online.

Virulence Genes

The presence of seven different extraintestinal virulence determinants (*sfa/foc, pap, afa, hly, cnf, aer,* and *ibeA*) was previously determined by polymerase chain reaction (PCR) (Picard et al. 1999).

Pathogenicity

The intrinsic extraintestinal virulence of the strains was studied by scoring the lethality in a mouse septicemia model as described previously (Picard et al. 1999; Johnson et al. 2006). Briefly, ten mice were inoculated by 10^8 colony forming units of bacteria subcutaneously in the neck and were observed for 7 days. In this model, the K-12 MG1655 strain does not kill mice whereas the ExPEC strain CFT073 kills all the inoculated mice (Johnson et al. 2006).

Media, Antibiotics, and Growth Conditions

Strains were grown in LB. Antibiotics and concentrations (in µg/ml) were ampicillin (Amp, 100), kanamycin (Kan, 30), gentamicin (Gm, 10), tetracycline (Tet, 10), and rifampicin (Rif, 100). Human urine was collected from several healthy male donors who were not taking any medication, pooled, filtered, and stored at −20 °C before use.

Construction of Plasmids and Strains

Construction of plasmids pRhomo (supplementary fig. S1, Supplementary Material online) and strains MG1655 *att*Tn*7::tetA1-kan-tetA2* and MG1655 *att*Tn*7::tetA1-kan-tetA2 recA* (supplementary fig. S2, Supplementary Material online) is described in detail in Supplementary Material online.

Estimation of RifR Mutant Frequencies

The mutant frequencies of 160 strains were estimated by monitoring their capacity to generate mutations conferring RifR in at least three independent clones for each strain. Between 10^2 and 10^3 cells from an overnight culture were inoculated into LB tubes and grown with shaking 24 h at 37 °C. Appropriate dilutions of the culture were plated onto LB petri dishes containing rifampicin (100 µg/ml) and incubated for 24 h at 37 °C. Mutant frequencies were

Table 1. *Escherichia coli* K-12 Strains and Plasmids Used in This Work.

K-12 Strain	Genotype/Phenotype	Origin/Reference
DH5α	*Δ(lacZ)M15, recA1, endA1, hsdR17, supE44*	Laboratory stock
MG1655	K-12 wild-type strain	Laboratory stock
MG1655 *recA*	MG1655 *recA938*::Tn*9–200*	Wertman et al. 1986
ME12	MG1655 *lacZ* Δ3′-*lacZ* Δ5′-*yfp*	Elez et al. 2007
ME12 *recA*	ME12 Δ *recA::Kan* (KmR)	Elez et al. 2007
Plasmid	**Main Characteristics/Resistance**	**Origin/Reference**
pGEM-T	Cloning PCR products; AmpR	Promega, USA
pGEM-T Easy	pGEM-T variant; AmpR	Promega, USA
pUC19	Cloning vector; AmpR	Invitrogen, USA
pBBR1MCS3	Cloning vector; TetR	Kovach et al. 1995
pBBR1MCS5	Cloning vector; GmR	Kovach et al. 1995
pUCGm*lox*	Vector with *aacC1*; GmR	Quenee et al. 2005
pRhomo	pGEM-T easy with *tetA* Δ5′-*aacC1-tetA*Δ3′; AmpR, GmR	This study
pGRG36	Contains Tn7 transposition machinery, a thermosensitive origin of replication and an origin of conjugation; AmpR	McKenzie and Craig 2006
pTetKanTet	A pGRG36 derivative which contains two incomplete fragments of the *tetA* resistance gene interspaced by a kanamycin resistance gene (*tetA1-kan-tetA2*); KmR, AmpR	This study

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calculated as the median number of rifampicin resistant colonies divided by the median number of viable colonies on LB-agar plates without antibiotic.

Estimation of TetR Recombinant Frequencies

In total, 160 strains (90 commensal and 70 pathogens) were transformed with plasmid pRhomo. Three independent transformants from each strain were purified onto LB-agar plates containing ampicillin and gentamicin and allowed to grow overnight at 37 °C. To diminish the probability of inoculating cells that contain plasmids already recombined, between 10^1 and 10^2 cells from each purified transformant were inoculated into LB with ampicillin but not gentamicin. Cultures were grown overnight with shaking until saturation. Appropriate dilutions were plated onto LB agar containing ampicillin and tetracycline and incubated for 24 h. TetR recombinant frequencies were calculated as the median number of recombinants (AmpR and TetR) divided by the median number of viable on LB-agar plates containing only ampicillin.

MG1655 *att*Tn*7*::*tetA1-kan-tetA2*, MG1655 *recA att*Tn*7*::*tet A1-kan-tetA2*, *E. coli* B, HS, F11, 536 and CFT073 either pRhomo-transformed or with the *att*Tn7 inserted construction, and a subset of 22 pRhomo-transformed strains were processed similarly: 10^2 cells from three independent overnight cultures were inoculated in parallel in LB-Amp or urine-Amp.

Statistical Analysis

Shapiro–Wilk test for normality was conducted for all distributions. When possible, parametric Student's *t*-test was applied to log-transformed data. When normality was not assessed, Mann–Whitney *U* test for nonparametric data was chosen for comparisons between distributions. After a significant Kruskall–Wallis test, Wilcoxon signed rank test with Bonferroni correction was used for multiple comparisons. To assess correlation between two variables, Pearson's product–moment or Spearman's rank correlation tests were used for lineal and monotonic relationships, respectively. Fisher's exact test was conducted to test for differences in the number of mutators on each group. Statistical test with *P* values under 0.05 was considered to be significant. All statistical analyses were performed using R software (http://www.R-project.org, last accessed March 23, 2015).

Supplementary Material

Supplementary methods, references, figures S1–S7, and tables S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

This work was supported by the Ministerio de Economía y Competitividad, Instituto de Salud Carlos III, the Spanish Network for Research in Infectious Diseases RD12/0015/0029, and Fondo de Investigación Sanitaria Grant PI13/00063. J.T. was supported by a grant from the Fondation pour la Recherche Médicale. The authors are grateful to Nicolas Plault for technical assistance and Michael McConnell for useful scientific comments and English revision of the manuscript.

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4. La sepiolita, un suplemento para piensos animales, promueve la transferencia horizontal de plásmidos.

Previamente al desarrollo de los trabajos que se presentan a continuación, en el grupo de investigación había surgido interés por el método de transformación bacteriana basado en el efecto Yoshida. De hecho, adaptamos y desarrollamos un protocolo específico para *P. aeruginosa* (45). La transformación mediante esta técnica se basa en que algunas arcillas porosas como la sepiolita son capaces de adsorber moléculas de DNA y, si se aplica una fuerza de fricción, forman complejos que pueden atravesar las membranas bacterianas, liberando en su interior el DNA con el que se había incubado previamente. Como método de transformación resulta conveniente pues requiere muy poco procesamiento y, aunque ofrece una eficiencia menor que otros protocolos más laboriosos, resulta útil para la transformación rutinaria en el laboratorio.

El primer artículo de la presente sección versa sobre el descubrimiento posterior de que la sepiolita es capaz de, no sólo transformar plásmidos libres en el medio, sino de mediar la transferencia directa de plásmidos entre dos células bacterianas. Mediante el uso de los marcadores de selección apropiados, se demuestra que la transferencia de plásmidos entre diferentes especies bacterianas sólo requiere la presencia de sepiolita, fuerzas de fricción y, obviamente, una bacteria donadora y una receptora.

Este fenómeno, que podríamos denominar *transferencia horizontal abiótica*, podría considerarse una mera curiosidad de laboratorio de no ser porque tiene unas implicaciones potencialmente muy relevantes, que se discuten en el segundo artículo.

La sepiolita es un aditivo muy común para piensos animales en la industria pecuaria ya que mejora la calidad y cantidad de carne. El tracto intestinal del ganado reúne todas las condiciones necesarias para que ocurra la transferencia mediada por sepiolita: alta concentración de especies bacterianas, fuerzas de fricción ocasionadas por los movimientos peristálticos y presión selectiva proporcionada por el uso extensivo de

antibióticos. De hecho, el ganado ha sido señalado por las autoridades como un importante reservorio de genes de resistencia, poniendo el foco en el uso de antibióticos como promotores del crecimiento. Los resultados presentados a continuación señalan que existen mecanismos abióticos de HGT y que aditivos no antibióticos como la sepiolita podrían estar fomentando la diseminación de determinantes de resistencia y virulencia.

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The Animal Food Supplement Sepiolite Promotes a Direct Horizontal Transfer of Antibiotic Resistance Plasmids between Bacterial Species

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Animal fodder is routinely complemented with antibiotics together with other food supplements to improve growth. For instance, sepiolite is currently used as a dietary coadjuvant in animal feed, as it increases animal growth parameters and improves meat and derived final product quality. This type of food additive has so far been considered innocuous for the development and spread of antibiotic resistance. In this study, we demonstrate that sepiolite promotes the direct horizontal transfer of antibiotic resistance plasmids between bacterial species. The conditions needed for plasmid transfer (sepiolite and friction forces) occur in the digestive tracts of farm animals, which routinely receive sepiolite as a food additive. Furthermore, this effect may be aggravated by the use of antibiotics supplied as growth promoters.

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There exists widespread concern about the massive amounts of antimicrobials used as growth promoters in livestock fodder and their relationship with the development of antibiotic resistance. Antibiotic use in animal husbandry may result in the selection of resistance determinants that can spread to human pathogens via different ways (1–3). The exposure of bacteria to antibacterial agents results in the selection of preexisting resistant variants that augment the likelihood of pathogen survival. Moreover, many antibiotics increase the mutation and recombination rates of bacteria, raising the probability of evolving resistance to even unrelated antibiotics (4). Finally, antibiotics can promote DNA sequence incorporation from other organisms via horizontal gene transfer (HGT) (5). HGT plays a major role among the mechanisms that control pathogen evolution, allowing bacteria to evade immunological responses, distribute genes that increase virulence, or acquire increased resistance to antibiotics (5). The majority of antibiotic resistances are most probably gained by lateral transfer of resistance genes from other bacterial strains or species (6).

It seems clear that the use of antibiotics in livestock, if not regulated, can be a dangerous source of antibiotic resistance determinants for human pathogens. However, animal feed is routinely complemented not only with antibiotics but also with other food supplements to enhance animal growth. For instance, sepiolite, which was authorized by the European Union in 1990 and registered as a technological additive for animal feed (E-562), is used as a dietary coadjuvant in fodder since it most likely reduces the speed of food passage through the intestinal tract, therefore allowing domestic animals to carry out a more efficient digestion of proteins. This results in an increase in parameters related to animal growth and in an improvement of meat and derived final product quality. Currently, sepiolite is used widely as a feed additive supplied to broiler chickens (7) and pigs (8), among other types of livestock. Furthermore, sepiolite has also been proposed as a remediation agent for contaminated soil (9). Up to the present, the use of this type of food additives has so far been considered innocuous for the development and spread of antibiotic resistance.

Recently, a DNA transformation method based on clay materials was described for bacteria (10, 11). The principle behind this method relies on minerals that, like sepiolite, are able to adsorb DNA and form nanoneedles, which, by the action of friction forces, are able to release and incorporate DNA into bacterial cells (12, 13). The procedure is able to transfer free DNA plasmids between bacterial species (11, 14, 15). Taking together the antibiotic selective pressure exerted on the livestock microbiome and the potential of sepiolite to transform bacterial cells, we studied whether sepiolite could mediate HGT between bacteria without the necessity of providing free DNA plasmids, i.e., could exert a direct transfer between bacteria. Based on the results presented here, we discuss the possibility that sepiolite may induce the transfer of resistance determinants in the digestive tracts of farm animals.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains used as donors in this study were *Escherichia coli* K-12 strain BW25113 (16), *E. coli* B strain BL21 (Invitrogen), and *Pseudomonas aeruginosa* strain PAO1 (17). Recipient strains were *E. coli* K-12 strain BW25113 *dinF*::Kan (16, 18), *E. coli* AB1155 *galK*::bla_{TEM1} (19), *Salmonella enterica* serovar Typhimurium strain SL1344 *nagZ*::Kan (Kan^r) (20), *P. aeruginosa* strain PAO1 (17), and *Mycobacterium smegmatis* strain mc^{2}155 (21). Table 1 shows the bacterial strains, plasmids, and antibiotic selection schemes used in the transformation experiments. The nonconjugative plasmids (nonautotransferring) used for the transfer experiments consisted of pCA24N (conferring chloramphenicol resistance [Cm^r]) (22), pGEM-T (conferring ampicillin resistance [Ap^r]) (Promega), pUCP24 (23) and pBBR1MCS5 (24) (both conferring gentamicin resistance [Gm^r]), and pVV16 (conferring hygromycin resistance [Hyg^r]) (25). All plasmids were previously introduced into the donors by electroporation.

In all cases, Luria-Bertani medium (LB; 10 g/liter of tryptone, 5 g/liter of yeast extract, and 5 g/liter of NaCl) was used. Solid LB plates were prepared with 2% agar, as previously described (14). Media were supple-

Received 27 November 2012 Returned for modification 28 December 2012
Accepted 19 March 2013
Published ahead of print 25 March 2013
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doi:10.1128/AAC.02363-12

TABLE 1 Strains, plasmids, and selection schemes used in transformation experiments

Donor	Receptor	Plasmid (antibiotic marker)	Donor counterselection agent (concn [μg/ml])
E. coli K-12 BW25113	*E. coli* K-12 BW25113 *dinF*::Kan	pCA24N (Cm)	Kan (50)
	Salmonella SL1344 *nagZ*::Kan	pCA24N (Cm)	Kan (50)
	P. aeruginosa PAO1	pUCP24 (Gm)	Kan (50)
	M. smegmatis mc²155	pVV16 (Hyg)	Nal (40)
E. coli B BL21	*E. coli* K-12 BW25113 *galK*::kan	pGEM-T (Amp)	Kan (50)
	Salmonella SL1344 *nagZ*::Kan	pGEM-T (Amp)	Kan (50)
	P. aeruginosa PAO1	pBBR1MCS5 (Gm)	Kan (50)
P. aeruginosa PAO1	*E. coli* K-12 AB1157 *galK*::*bla*	pUCP24 (Gm)	Cb (500)

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mented with antibiotics at the following concentrations: kanamycin (Kan) at 50 μg/ml, chloramphenicol (Cm) at 40 μg/ml, nalidixic acid (Nal) at 40 μg/ml, gentamicin (Gm) at 30 μg/ml, ampicillin (Ap) at 100 μg/ml, carbenicillin (Cb) at 500 μg/ml, and hygromycin (Hyg) at 100 μg/ml.

Plasmid transfer experiments. About 5×10^8 bacterial cells of donor (containing the appropriate plasmid) and recipient strains from overnight cultures were centrifuged and resuspended in 100 μl of sterilized transformation mixture, consisting of sepiolite (Kremer Pigmente, Spain) suspended in aqueous solution at a final concentration of 100 μg/ml.

Resuspended cells were spread on plates containing fresh LB medium solidified with 2% agar, and petri dishes were predried in a biological safety flow cabinet for 20 min before use. Friction force was provided by streaking bacterial cultures plus sepiolite with sterile glass stir sticks gently pressed onto the medium surface for 1 min, applying as much pressure as possible without breaking the agar gel. Petri dishes were incubated at 37°C for 1 h (2 h in the case of recipient *M. smegmatis*) to allow for antibiotic gene marker expression. The respective antibiotics to select for transformants (recipients containing the plasmid) and counterselect for donors were added to each plate, in a volume of 250 μl uniformly spread onto the surface of the medium by use of glass beads. Plates were incubated overnight (for 36 h when *M. smegmatis* was used as a recipient). Every experiment was carried out in quadruplicate and repeated at least twice. Transfer of plasmids was verified by extraction from some transformants of plasmid DNA, followed by restriction analysis. Transfer efficiencies were expressed as numbers of transformants per donor cell.

RESULTS AND DISCUSSION

In order to prove the idea that plasmid DNA can be transferred between bacteria without previous plasmid extraction, we designed a simple laboratory experiment: a mixture consisting of *E. coli* K-12 strain BW25113 harboring plasmid pCA24N (nonautotransferring and conferring Cm^r) and *E. coli* BW25113 *dinF*::Kan (Kan^r) (harboring no plasmids and resistant to kanamycin [with resistance encoded by a chromosomal marker]) was treated with friction forces (i.e., spreading with sterile glass stir sticks gently pressed onto the medium surface, applying as much pressure as possible without breaking the agar gel) in the presence of sepiolite for 1 min. One hour after the application of the friction forces, plates were supplemented with kanamycin (50 μg/ml) to counterselect the donor strain and with chloramphenicol (40 μg/ml) to select for the transformants (recipients containing the plasmid). The results clearly showed that the plasmid was easily transferred to the *E. coli* recipient strain by the joint action of the friction forces and sepiolite. An average transfer efficiency of 5×10^{-6} transformant per recipient cell was obtained (Fig. 1). Control experiments followed an identical protocol yet omitted sepiolite.

To test if sepiolite-based plasmid transfer may also occur with regard to other species, we used the same donor strain, BW25113, containing plasmid pCA24N (Cm^r), pUCP24 (Gm^r) (23), or pVV16 (Hyg^r) as a donor and *S. enterica* serovar Typhimurium strain SL1344 *nagZ*::Kan (Kan^r), *P. aeruginosa* strain PAO1, or *M. smegmatis* strain mc²155 as the recipient, respectively. Following the same procedure as that described above and selecting transformants with the appropriate antibiotics, we obtained average transfer efficiencies of 5×10^{-8}, 5×10^{-7}, and 1×10^{-7} transformant per receptor cell for *S. enterica*, *P. aeruginosa*, and *M. smegmatis*, respectively (Fig. 1).

In order to elucidate whether this plasmid transfer was exclusive to *E. coli* K-12, we also used another *E. coli* strain of different origin, *E. coli* B strain BL21, as a donor. Plasmid pGEM-T (Ap^r) was transferred from the *E. coli* B strain to *E. coli* K-12 strain BW25113 *dinF*::Kan (Kan^r) and to *S. enterica* serovar Typhimurium strain SL1344 *nagZ*::Kan (Kan^r). Plasmid pUCP24 was used to test the transfer from *E. coli* B strain BL21 to *P. aeruginosa* strain PAO1. Plasmids were transferred in all cases to the different recipients, at frequencies of 3.2×10^{-6}, 1.2×10^{-8}, and 1.7×10^{-8} for *E. coli* K-12 *dinF*::Kan, *S. enterica* serovar Typhimurium strain SL1344 *nagZ*::Kan, and *P. aeruginosa* strain PAO1, respectively (Fig. 1).

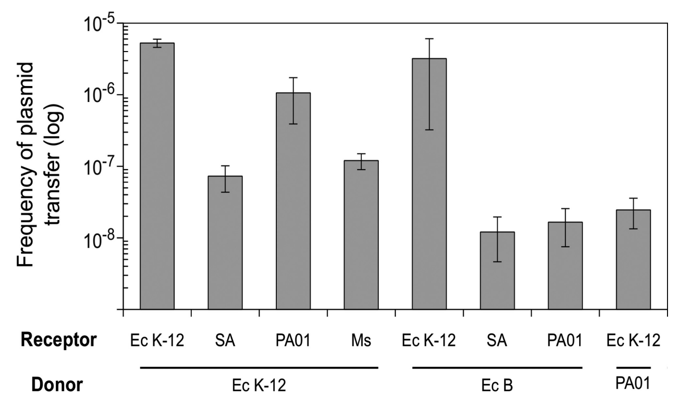

FIG 1 Efficiencies of direct plasmid transfer. Plasmid transfer was monitored for the following species and strains: *E. coli* K-12 (Ec K-12), *E. coli* B (Ec B), *S. enterica* serovar Typhimurium (SA), *M. smegmatis* mc²155 (Ms), and *P. aeruginosa* PAO1 (PA01). Plasmids used consisted of pCA24N and pGEM-T for *E. coli* and *Salmonella*, pUCP24 and pBBR1MCS5 for *P. aeruginosa* PAO1, and pVV16 for *M. smegmatis*. Data show the means for four experiments. Error bars represent standard deviations.

Finally, to test if other species different from *E. coli* could act as plasmid donors by this procedure, we performed the same experiment using *P. aeruginosa* strain PAO1 as the pUCP24 plasmid donor and *E. coli* K-12 strain AB1157 *galK*::Kan as the receptor. Again, plasmids were successfully transferred at a frequency of 2.5×10^{-8} (Fig. 1).

Most importantly, the control experiments in the absence of sepiolite produced no colonies in any of the cases (efficiencies of $\leq 2 \times 10^{-9}$ transformant per receptor cell). Therefore, our data clearly indicate that the transfer of plasmid DNA was due only to the sepiolite-mediated transformation process, ruling out conjugation, competence, or *de novo* mutations, and thus confirming that sepiolite promotes direct HGT between bacteria.

Recently, Yoshida and Fujiura proposed that seismic movements may promote bacterial transformation in natural clay substrates (26). Our findings demonstrate that this transfer may occur directly from bacterium to bacterium, as friction forces generated in the interface of ground material may equally promote bacterial genetic material release and transformation. We propose here the most probable environment for sepiolite-mediated DNA transfer between bacteria: the digestive tracts of farm animals. All the necessary ingredients that allow for a direct transfer of plasmid DNA between bacteria occur in farm animals: routine administration of antibiotics, sepiolite used as a food additive, pathogenic and commensal bacteria sharing the same niche, and the mechanical friction provided by peristalsis in the rumen or intestines, if not the strong abrasive action of the gizzard in poultry.

The results presented here demonstrate that a direct genetic material exchange among different strains and species is promoted by sepiolite. This should strongly encourage the scientific community working on antibiotic resistance to start studying this phenomenon *in vivo*, undertaking the appropriate experiments in livestock.

Finally, it has not escaped our attention that this finding provides a new laboratory method suitable for direct plasmid transformation between bacterial strains or species, without having to resort to plasmid isolation and competent cell preparation.

ACKNOWLEDGMENTS

This work was supported by the Instituto de Salud Carlos III, cofinanced by the European Regional Development Fund "A Way to Achieve Europe," by the Spanish Network for Research in Infectious Diseases (grant REIPI RD06/0008), and by grant PI10/00105 (FIS-ISCIII) and PAR project 241476 of the EU 7th Framework Programme.

We thank Paul Johnston from the Freie Universität in Berlin for his comments on the manuscript.

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Antibiotics **2015**, *4*, 299-308; doi:10.3390/antibiotics4030299

OPEN ACCESS

antibiotics

ISSN 2079-6382

www.mdpi.com/journal/antibiotics

Hypothesis

Can Clays in Livestock Feed Promote Antibiotic Resistance and Virulence in Pathogenic Bacteria?

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Academic Editor: Martin J. Woodward

Received: 3 June 2015 / Accepted: 13 July 2015 / Published: 16 July 2015

Abstract: The use of antibiotics in animal husbandry has long been associated with the appearance of antibiotic resistance and virulence factor determinants. Nonetheless, the number of cases of human infection involving resistant or virulent microorganisms that originate in farms is increasing. While many antibiotics have been banned as dietary supplements in some countries, other additives thought to be innocuous in terms of the development and spread of antibiotic resistance are used as growth promoters. In fact, several clay materials are routinely added to animal feed with the aim of improving growth and animal product quality. However, recent findings suggest that sepiolite, a clay additive, mediates the direct transfer of plasmids between different bacterial species. We therefore hypothesize that clays present in animal feed facilitate the horizontal transfer of resistance determinants in the digestive tract of farm animals.

Keywords: antimicrobial; antibiotic resistance; horizontal gene transfer; virulence factor; clay; sepiolite

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1. Introduction

The exposure of bacteria to inhibitory concentrations of antibiotics results in the selection of resistant sub-populations. In addition, sub-inhibitory levels of many antibiotics raise mutation and recombination rates in bacteria, increasing the probability of resistance acquisition, even to unrelated antibiotics [1,2]. Antimicrobial agents also promote the acquisition of DNA sequences from other organisms via horizontal gene transfer (HGT) [1,3]. HGT plays a major role in pathogen evolution by allowing bacteria to evade the immune response and distributing genes that increase virulence or provide increased resistance to antibiotics [4]. Indeed, there is now much concern surrounding the huge quantity of antimicrobial agents used as livestock growth promoters and the possibility that they select for resistance determinants, which can spread by different routes to human pathogens compromising antibiotic efficacy [5,6]. Furthermore, antibiotic resistance genes conferring resistance to several antimicrobials have been used for the construction of some genetically modified plants which are used as animal feed [7]. The possibility of gene transference via HGT to the microbiota and subsequently to the community must therefore be examined [8].

Animal feed also contains other supplements such as certain clays. Sepiolite, for example, was authorized for use by the European Union in 1990 and registered as technological additive E-562. The purpose of these minerals is to make digestion more efficient. These additives are believed to improve growth and animal product quality, and are widely used in feed for broiler chickens [9] and pigs [10]. Such practices are considered innocuous. Clays, including sepiolite, have also been proposed for use in a large number of pharmaceutical applications [11,12]. Interestingly, due to their excellent adsorption capacities, clays have been proposed as carriers for antibiotics among other compounds [11]. Furthermore, certain clays present some antibacterial effects, due to the presence of bactericidal metallic cations (mostly Fe^{2+} or Cu^{2+}) [13] either of natural origin [14], or adsorbed artificially [15]. Scientific literature states that they fulfill all safety, stability, and chemical inertia requirements, and their presence in tablets used in human medicine has been established as safe and without side effects [11].

A relationship between the presence of sand or clay minerals and bacterial genetic exchange has been reported, and the possibility that mineral-mediated DNA transfer between different bacterial species plays a role in the evolution of antibiotic resistance has received some attention [16]. Transformation of bacteria by foreign DNA in the presence of friction forces and clay materials is known as the Yoshida effect [17]. This transformation relies on the ability of mineral nanofibers (or nanoneedles), such as those formed by sepiolite, to adsorb DNA and form a chestnut burr-shaped complex when a sliding friction force [18] or even vibrations [16] are applied. These complexes can penetrate the bacterial cell wall and membrane, releasing DNA into the cytoplasm [19]. This effect was observed in many bacterial species (Table 1).

Table 1. Bacterial species in which clay-mediated transformation has been examined.

Bacterial Species	References
Pseudomonas aeruginosa	[20]
Salmonella enterica Serovar Typhimurium	[20]
Mycobacterium smegmatis	[20]
Pseudomonas putida	[21]
Escherichia coli (several strains)	[21–23]
Yersinia enterocolitica	[23]
Acinetobacter baumannii	[23]
Bacillus subtilis	[16]
Pseudomonas elodea	[16]

Here we propose that "recipient" bacteria can be transformed by DNA fragments or plasmids from "donor" bacteria adsorbed onto mechanically-penetrating mineral nanofibers. Laboratory work has already provided proof of concept of this idea; the exclusive combination of friction forces and sepiolite allows plasmids to be easily transferred between bacterial species, including strains of *Escherichia coli*, *Salmonella enterica* (serovar Typhimurium), *Pseudomonas aeruginosa* and *Mycobacterium smegmatis* [24]. These experiments were designed in such a way that different antibiotic-resistance markers could be used to counterselect donor bacteria. The results clearly demonstrated that DNA transfer between bacteria was mediated by sepiolite.

2. The Hypothesis

The combination of clays present in animal feed, the presence of both pathogenic and commensal bacteria in the animal gut, the mechanical friction provided by peristalsis in the rumen and intestines (or the strong abrasive action of the gizzard in poultry), and the routine administration of antibiotics to livestock (providing the selective force required for the fixation of antibiotic resistance), led us to hypothesize that clay-mediated DNA transfer among bacteria may occur in farm animals. This phenomenon could provide an additional HGT mechanism able to disseminate antibiotic resistance and virulence genes across species (Figure 1).

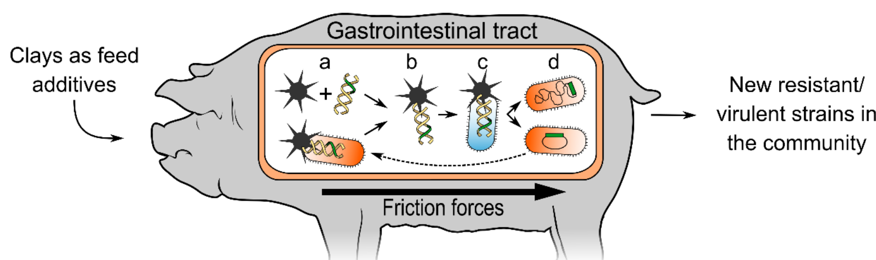

Figure 1. Clay-mediated DNA transfer in livestock. Clay additives in animal feed form complexes due to friction forces that may promote the release of DNA from bacteria. This free DNA can then be adsorbed by clay (a). The resulting DNA-clay complexes (b) penetrate other microbes, internally delivering the carried DNA (c). This may result in the acquisition of new virulence genes and/or antibiotic resistance determinants (d). Both commensal and pathogenic bacteria may act as recipients or donors and acquire traits that compromise the treatment of infections.

The presence of antibiotics would not only provide the selective pressure to fix transformed cells in their populations, but is also an additional source of DNA via cell lysis. Plasmid DNA, which does not need to integrate into a recipient bacterium's chromosome, might be efficiently introduced by clay-mediated transfer. Mobile elements such as transposons or integrons also become active in the presence of antibiotics [25] and could be transferred with the help of clays. The fact that antibiotics increase rates of exogenous DNA recombination (*i.e.*, the frequency of integration of foreign DNA into the bacterial chromosome) [4,26] might indeed improve the likelihood of transformation.

Clay-mediated transfer of DNA have been experimentally demonstrated *in vitro* and the parameters necessary for its occurrence are relatively well characterized [17,22,27]. Despite the obvious differences with the environment found in the laboratory, the key conditions required for clay-mediated transference could be found in the gastrointestinal tract of farm animals. For example, the optimum temperature for DNA adsorption by clay materials is 37 °C [27], which is within the body temperature range of cattle. The friction forces required for transformation driven by clay-DNA complexes are common in the gastrointestinal tract of livestock. *In vitro*, an optimum transformation pressure of 3.9 kPa has been recorded [22]; such a pressure could easily be achieved in the gastrointestinal tract of most farm animals (Table 2).

Table 2. Pressure values exerted in different parts of the digestive tract of several livestock species. The optimal pressure for the clay-mediated transformation of bacteria by plasmid DNA is reported to be 3.9 kPa [22]. The numbers shown have been converted to the international system unit for pressure.

Average Pressure of Digestive Tract (kPa)	Species	Digestive Tract Section	References
2.7	humans	jejunum	[28]
2.7	humans	pylorus	[29]
10.7	humans	oesophagus	[30]
37.3	geese	gizzard	[31]
3.1	turkeys	muscular stomach	[32]
0.5–39.9	chickens	gizzard	[33]
3.9	chickens	muscular stomach	[32]
4.7	pigs	stomach	[34]

The gut environment is generally characterized by a low pH and an elevated osmolarity. These circumstances could, in principle, hamper DNA transfer, but clay-mediated transformation has been shown to be robust to such conditions as clay materials can adsorb DNA over a wide range of pH. Since DNA stability is compromised at highly acidic conditions, clay-adsorbed DNA is unable to transform bacterial cells at pH lower than 3 [27]. However, pH values found in the stomach of mammals and the gizzard of poultry are high enough (4.4 in pigs [35] and 3.5 in broiler chickens [36]) to allow DNA adsorption and transformation [27]. Furthermore, the pH in the intestine of livestock rises up to ~6 [35,36], a closer value to the adsorption and transformation optimum found *in vitro* [27]. Regarding salinity, clay-mediated transformation has been reported to occur at a range of 0–0.3 M of NaCl without a significant loss of efficiency *in vitro* [17]. The osmolarity in the gut, based on a complex mixture of salts, has been estimated to be equivalent to 0.3 M NaCl [37], which is, again, within the

limits for optimal transformation *in vitro*. Taken together, the confluence of *in vitro* and *in vivo* data suggests that gastrointestinal tract is a plausible scenario for clay-mediated DNA transfer.

One might argue that if free DNA fragments were present in the digestive tract of animals, they would be rapidly degraded. On the other hand, it has been shown that clay-adsorbed DNA is very resistant to degradation in the gut and soil [16]. Additionally, it has been reported that DNA adsorption by clays provides protection against nucleases [27,38]. However, this protection seems to be only partial: it has been shown that clay-adsorbed plasmidic DNA treated with DNase I displayed a restricted availability for transformation, suggesting that DNA molecules were not intact. Yet, even partially degraded DNA could still serve as a substrate for integration into the recipient chromosome by recombination [38]. This protected DNA therefore provides a reservoir of genetic material that could benefit naturally-occurring competent bacteria in the digestive tract and allow clay-mediated transformation.

Previously discussed arguments and laboratory results obtained to date, provide sufficient evidence to recommend examining clay-mediated DNA transfer *in vivo*. Thus, we propose that the role of sepiolite and other clays as promoters of antibiotic resistance and virulence trait transfer in farm animals should be investigated. To what extent the proposed mechanism of clay-mediated HGT could operate *in vivo* is difficult to answer. Conjugation consists of transferring DNA fragments ranging from a few base pairs to large chromosomes or plasmids. The process of conjugation requires cell-to-cell contact, mating pair formation and transfer of plasmid DNA through a conjugative pilus. The genes for conjugative machinery are encoded by autonomously replicating plasmids or by integrative conjugative elements in the chromosome [39]. Conjugation rates in literature tend to vary a lot. One of the highest conjugation rates observed for plasmids is around $\sim 10^{-3}$ transconjugants per recipient per hour in bacterial biofilms *in vitro*. According to the authors, these values are from one to three orders of magnitude greater than those reported by other studies [40], while the picture *in vivo* is slightly different. Conjugation rates have been examined using mice, yielding highly dissimilar results that vary from very low frequencies [41] to as high as $\sim 7 \times 10^{-1}$ transconjugants per donor cell, a value that was much higher than the rate found *in vitro* ($\sim 6 \times 10^{-8}$) using the same strains [42]. These results suggest that the *in vitro* measured rates of genetic exchange are poor estimators of the *in vivo* frequencies.

In our previous experiments we found frequencies ranging from 10^{-6} to 10^{-8}, which are comparable to most of conjugation experiments [24]. However, the experiments were carried out applying friction forces during one minute only. In the intestinal tract, discontinuous, but frequent friction forces of different intensities and duration are expected. Then, our *in vitro* results may underestimate the frequencies of this atypical HGT if it indeed occurs *in vivo*. Nevertheless, even very low frequencies of DNA transfer should be taken into consideration, as the possible consequences for human and animal biosafety could be of great importance. Furthermore, risk assessment should not be based solely on the observed frequencies, as they are not good predictors of long-term effects on bacterial communities [43].

3. Testing the Hypothesis

An ideal testing framework for our hypothesis may consist in a double blind, stratified experiment in which four groups of gnotobiotic animals are given feed containing or lacking sepiolite, an appropriate bacterial donor strain and antibiotics acting as selective force (Figure 2).

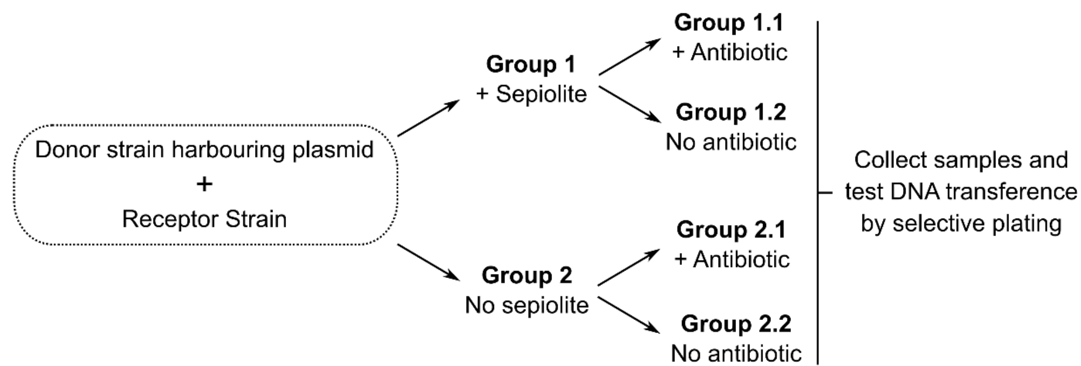

Figure 2. Ideal experimental model for testing the stated hypothesis. All germ-free animals in all groups should be given feed containing a safe bacterial strain harboring a resistance marker integrated in the chromosome or a known plasmid that cannot be transferred by conjugation (non-conjugative and non-mobilizable ones); this bacterium should carry an auxotrophy to allow for the counterselection of donors. A known recipient (for instance an *E. coli* strain different from the donor) could be used to easily verify transfer.

Faecal samples would then be collected to isolate bacteria in a medium designed to counterselect donor strains. Differences in the transfer of resistance genes would reflect the effect of the clay. Available reports on the efficiency of sepiolite in promoting bacterial transformation [24], the high sepiolite content of animal feed and the high bacterial content in faecal samples; it is expectable that even at low *in vivo* transfection efficiency and wide margins of error, this hypothesis might be accurately tested in a cost-effective way. However, a testing scenario would be strongly limited by the fact that experiments would require an S1 security level, and this is difficult to achieve with livestock animals. We should also admit that it is enormously difficult to detect rare HGT events and the resulting bacterial transformants within large heterogeneous microbial communities [44], which confer a poor resolution to this experimental approach. One possibility is the use of an animal model like in one study reporting vancomycin resistance gene transfer between enterococci of human origin in the gut of mice harbouring human microbiota [45]; although this would only provide an indirect evidence for livestock in case of positive results.

Epidemiological observations correlating the use of clay supplements with higher incidence of antibiotic resistance can be useful to initiate additional studies. In near future, the possibility of doing large comparative metagenomic studies in the microbiome of livestock may help to correlate the use of clay with altered frequencies of HGT. If our hypothesis is correct, “remodeled” bacteria are being transmitted from farms to communities. Clay materials in animal feed perchance significantly contribute to the spread of antibiotic resistance and virulence genes, increasing the problems of antibiotic ineffectiveness in the treatment of human and animal infections. This new mechanism of HGT may promote the acquisition of virulence factors and antibiotic resistance by gut pathogens with broad implications for humans and animals.

4. Conclusions

Here, we present a hypothesis raising the concern that under a livestock feeding regime, the confluence of factors such as antibiotic pressure, diversity and size of bacterial populations, the presence

of potential pathogens, the particular physiology of digestive tract of cattle and clay present as a feed additive might favor clay-mediated horizontal gene transfer. The kind of transferred materials may consist of plasmids, and more importantly fragments of genomic DNA that may spread both antibiotic resistance and virulence factors between potential animal and human bacterial pathogens and transmit them to the community. We encourage the scientists from related disciplines to be aware about this possible mechanism and perform additional studies to see to what extent this HGT is operating.

Acknowledgments

This work was funded by the *Instituto de Salud Carlos III* (*Ministerio de Economía y Competitividad*), the European Development Regional Fund (ERDF) (*A way to achieve Europe*), the Spanish Network for Research into Infectious Diseases (REIPI RD12/0015), grant PI10/00105 from the FIS-ISCIII, and the PAR project (Ref. 241476) from the EU 7th Framework Programme. JRV was supported by COST BM1006 and the CYTED 510RT0391 network. ARR was supported by The Collaborative Research Centre (CRC) via project 973, C5 "Priming and Memory of Organismic Responses to Stress". We are grateful to Jens Rolff, Paul Johnston, Guozhi Yu and Olga Makarova from Freie Universität Berlin for nice suggestions about the manuscript.

Authors Contributions

Alexandro Rodríguez-Rojas, Jerónimo Rodríguez-Beltrán, José Ramón Valverde and Jesús Blázquez conceived the hypothesis, analyzed the data and wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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"Scientists often have a naive faith that if only they could discover enough facts about a problem, these facts would somehow arrange themselves in a compelling and true solution"

Theodosius Dobzhansky
Mankind Evolving: The evolution of the Human Species

Capítulo V
Discusión

Todos los organismos vivos de este planeta disponen de mecanismos evolutivos cuya magnitud ha sido minuciosamente ajustada durante el transcurso del largo periodo que sobrevino a la aparición de la vida en la Tierra. Este ajuste fino permite que, en general, exista un balance preciso entre una estabilidad genética relativamente alta a nivel individual y una capacidad de generar diversidad y experimentar cambios adaptativos a nivel poblacional. El principal objetivo de esta tesis ha sido cuantificar y ampliar el conocimiento sobre los diferentes sistemas evolutivos implicados en el control de la tasa adaptativa en los seres vivos, usando para ello la bacteria modelo *E. coli*. La mutación, la recombinación y la transferencia horizontal de genes son los mecanismos más prominentes de la evolución bacteriana. Todos ellos han sido abordados (aunque desde diversas aproximaciones) durante el transcurso de este doctorado, en un humilde intento por ampliar el conocimiento sobre las raíces de la evolución, manteniendo en perspectiva el contexto de las infecciones bacterianas, los factores de virulencia que las potencian y el desarrollo de resistencia a los antibióticos que nos impiden acabar con ellas.

De hecho, la evolución de resistencia silenció la euforia inicial que acompañó al descubrimiento y posterior comercialización de los antibióticos y todavía silencia muchos de los esfuerzos en la lucha contra las enfermedades infecciosas. El estudio de las causas subyacentes a la aparición y diseminación de resistencias es, por tanto, un tema de vital importancia.

La visión clásica estipula que la exposición bacteriana agentes antimicrobianos tiene como principal consecuencia la selección de variantes resistentes preexistentes que sobrevivirán al tratamiento. Es decir, que la exposición a agentes selectivos no influye en la aparición de mutantes resistentes y por tanto la mutación es previa a la selección (160). Esta visión es esencialmente correcta pero descubrimientos más recientes hacen necesaria su actualización. Por ejemplo, los antibióticos pueden seleccionar indirectamente cepas hipermutadoras que tendrán mayores posibilidades de desarrollar

resistencia por mutación a futuros tratamientos, lo que se conoce como selección de segundo orden (161). Además, en la presente tesis y en trabajos anteriores (162–165) se ha demostrado que una amplia gama de antimicrobianos en concentraciones específicas pueden inducir una respuesta adaptativa mediada por mutación en las bacterias que los sufren. El hecho de que la mayoría de antibióticos que inducen mutagénesis inducen también la respuesta SOS (p. ej., ciprofloxacino, trimetroprima o β-lactámicos) es conocido desde hace tiempo (119, 120, 166). En consecuencia, se ha propuesto que la inactivación del sistema SOS podría tener efectos beneficiosos como terapia adyuvante al tratamiento antibiótico ya que reduciría la aparición de mutantes (153, 167). De hecho, se han desarrollado diferentes estrategias para encontrar moléculas capaces de inhibir RecA *in vivo* (152, 168, 169).

Sobre la inhibición de *recA* como terapia adyuvante

Nuestros principales objetivos en la primera parte de la tesis fueron, por un lado, determinar qué antibióticos (o al menos qué familias) son capaces de inducir mutaciones y en qué medida y, por otro lado, determinar cuál es el efecto de la inactivación de RecA y por consiguiente de la mutagénesis SOS.

Para ello, trece antimicrobianos diferentes, pertenecientes a distintas familias (β-lactámicos, quinolonas, inhibidores de la síntesis de folatos, polimixinas, tetraciclinas, aminoglucósidos, rifamicinas, fosfomicina y cloramfenicol) fueron utilizados para tratar durante cuatro horas cultivos exponenciales de *E. coli* y su mutante isogénico Δ*recA*. Los resultados de la frecuencia de mutantes realizada después de cada tratamiento demuestran que muchos antibióticos (ocho de trece en total) son capaces de inducir mutagénesis en la cepa silvestre, aunque en general el aumento es relativamente pequeño. Sin embargo, la inactivación de *recA* reduce la mutagénesis a los niveles de los cultivos control no tratados para todos los antibióticos (salvo para la ceftazidima), lo que indica que probablemente las polimerasas propensas a error controladas por el SOS estén involucradas en el aumento.

Los mecanismos moleculares que están detrás del incremento en mutagénesis están relativamente bien caracterizados y han sido objeto del apartado "Inducción del SOS mediada por agentes genotóxicos". Con respecto a los β-lactámicos, se ha descrito que la inhibición de la división que causan induce la expresión del operón *dpiBA*, un sistema de dos componentes cuyo efector, la proteína DpiA, se une al origen de replicación cromosómico impidiendo la replicación, lo que conlleva la inducción de la respuesta SOS (120). Además, los β-lactámicos promueven una bajada de la fidelidad replicativa mediante una disminución de los niveles de MutS (144) y la inducción de *dinB* de un modo independiente del SOS (170), lo que podría explicar que el incremento en la tasa de mutación causado por ceftazidima no sea exclusivamente *recA*-dependiente en nuestras condiciones experimentales.

Otros antibióticos inducen la respuesta SOS ya que su modo de acción está directamente relacionado con el metabolismo del DNA. Las quinolonas (151) y los inhibidores de la síntesis de folatos (trimetroprima y sulfametoxazol) (162) son un buen ejemplo. Las primeras, producen DSB al inhibir las topoisomerasas de tipo II por lo que, como se demuestra en nuestro estudio con la fusión transcripcional *recA*::GFP, son potentes inductores del sistema SOS. Según la mayoría de modelos al respecto, la reparación de este tipo de daños suele realizarse por recombinación, pero en ausencia de DNA homólogo o cuando los DSB son muy frecuentes se induce el SOS y, por tanto, las polimerasas propensas a error. Sorprendentemente, a pesar de ser uno de los mejores activadores del SOS, el ciprofloxacino sólo produce un aumento relativamente moderado (alrededor de dos veces) en la mutagénesis.

En cambio, los inhibidores de la síntesis de folatos producen el mayor incremento en la aparición de mutantes en nuestras condiciones experimentales. Estos antimicrobianos inhiben la síntesis del tetrahidrofolato, un precursor de las bases nitrogenadas sin el cual la célula es incapaz de sintetizar timina (118). A parte del bloqueo de la horquilla replicativa [y la consecuente inducción del SOS (119)] esto tiene como corolario

un desequilibrio del *pool* de nucleótidos, lo que puede tener un efecto aditivo con la expresión de las polimerasas propensas a error en la mutagénesis (171).

Cabe reseñar que nuestros resultados demuestran que la tetraciclina también produce un incremento en la frecuencia de mutantes. Sin embargo, en este caso no se ha detectado inducción del sistema SOS, por lo que resulta sorprendente que la inactivación de *recA* sea capaz de reducir la aparición de mutantes. La explicación de este fenómeno resulta elusiva, aunque se ha reportado que la tetraciclina puede ser mutagénica en células de ratón (172). Adicionalmente, el tratamiento prolongado (hasta cinco días) de *P. aeruginosa* con tetraciclina, causa un marcado incremento en la aparición de mutantes resistentes a dicho antibiótico (173), lo que ha sido interpretado como una señal de mutagénesis inducida por estrés. Sin embargo, la brevedad del tratamiento (tan sólo cuatro horas) hace que dicha posibilidad parezca poco probable en nuestras condiciones.

Otra posible explicación radica en el hecho de que en *E. coli* se ha descrito que concentraciones subinhibitorias de otros antibióticos, como la estreptomicina, cuyo mecanismo de acción es similar al de la tetraciclina (bloqueo de la síntesis proteica), pueden producir errores en la traducción. Cuando las proteínas encargadas de la replicación del DNA se ven afectadas, su fidelidad puede verse comprometida (174). De hecho, de un modo similar a nuestros resultados con tetraciclina, en determinadas cepas de *E. coli* se ha demostrado que tratamientos con estreptomicina provocan un fenotipo mutador dependiente de RecA (175).

En cualquier caso, los resultados presentados en esta tesis demuestran que efectivamente existe un potencial terapéutico en la inhibición de *recA* como adyuvante en la terapia antibiótica. Más aún si cabe, si tenemos en cuenta que RecA no sólo afecta a la mutagénesis, sino que su actividad determina una miríada de procesos fisiológicos con implicaciones clínicas como la sensibilidad a agentes antimicrobianos, la recombinación homóloga (71), el desplazamiento por *swarming* (176), la formación de *biofilms* (177), la

inducción de persistencia (178) o la patogénesis (179) entre otros procesos celulares.

El sistema SOS tiene como función principal proteger a la célula después de una agresión o daño en el DNA. En ocasiones, y como se ha comentado recientemente, esto puede tener como consecuencia el aumento en la tasa de mutación. Aunque este aumento puede ser provechoso a nivel poblacional ya que aumenta la probabilidad de encontrar mutaciones beneficiosas (como de resistencia a agentes antimicrobianos), resulta una maniobra muy arriesgada a nivel individual. Probablemente por ello, se han seleccionado estrategias para mantener una tasa de mutación relativamente baja en la mayoría de las condiciones y sólo en casos excepcionales (por ejemplo bajo un tratamiento antibiótico) y quizás sin ningún otro remedio, ocurre un relajamiento de la fidelidad replicativa. Se podría considerar que el sistema SOS tiene dos caras: En la primera, mucho más común, prima la reparación no mutagénica y en la segunda, que es comparativamente poco frecuente, aumenta la mutagénesis. Es precisamente la caracterización de un gen involucrado en el primer tipo de estrategia de lo que trata la segunda publicación de esta tesis de la que se comentarán a continuación algunos aspectos.

Sobre la caracterización del gen SOS *dinF*

El gen *dinF* fue uno de los primeros en ser identificados en el sistema SOS (129), sin embargo su función ha permanecido elusiva a la comunidad científica durante más de treinta años. El hecho de que aparentemente forme un una unidad transcripcional con *lexA* (155), el regulador maestro del regulón SOS, sugiere una regulación muy precisa y una inducción temprana, ya que *lexA* es uno de los genes que más rápido se liberan de la represión tras la inducción del SOS (180). Además, DinF es un miembro prototípico de la familia de bombas de expulsión MATE, caracterizadas por poseer doce dominios transmembrana (157) y mediar el antiporte de múltiples sustancias tóxicas (como antibióticos y colorantes) acoplado a cationes Na^+ o H^+ (181).

Previamente, el grupo de investigación había encontrado mediante un escrutinio

diseñado para encontrar genes antimutadores que la bomba MATE de *E. coli* NorM protege frente a estrés oxidativo, reduciendo la frecuencia de mutantes en un fondo Δ*mutT* a niveles de la cepa silvestre (156). Dado que la homología a nivel de secuencia entre DinF y NorM es significativa, que los sistemas de protección frente a ROS son, en general, redundantes (182) y que el daño oxidativo es un conocido inductor del sistema SOS (115) resulta razonable suponer que, quizás, una de las funciones de DinF sea la de proteger frente a este tipo de insulto.

Los experimentos que realizamos demuestran que, efectivamente, DinF está implicado en la supervivencia frente a peróxido de hidrógeno, ya que su eliminación reduce la viabilidad celular cerca de un orden de magnitud. Resulta reseñable que en un fondo carente del gen *mutT* la supervivencia celular se ve reducida aproximadamente dos logaritmos, y que la sobreexpresión de *dinF* desde un plásmido es capaz de rescatarla hasta niveles similares a la cepa silvestre.

Además, la sobreexpresión de *dinF* reduce los niveles de radicales libres del oxígeno producidos de forma endógena y la carbonilación de proteínas lo que señala que probablemente la protección observada frente a peróxido de hidrógeno esté mediada por una reducción de los ROS intracelulares.

Como se ha comentado en la introducción, los ROS resultan altamente mutagénicos, pues las bases nitrogenadas (especialmente la guanina) presentan un potencial redox bajo lo que les hace especialmente vulnerables a la oxidación (183). La forma oxidada de la guanina, la 8-oxo-Guanina, presenta una promiscuidad química que le permite emparejarse tanto con adenina como con citosina, por lo que si se da en el DNA suele ocasionar mutaciones (11). Para lidiar con este tipo de daño en *E. coli* existen una serie de proteínas especializadas entre las que se encuentra MutT. En consecuencia, en el mutante Δ*mutT* la tasa de mutación espontánea es entre cien y mil veces superior a la cepa silvestre. Sin embargo, la sobreexpresión de *dinF* es capaz de reducir al menos un logaritmo dicha tasa de mutación lo que, de nuevo, apunta a una capacidad para reducir

en el estado oxidativo intracelular.

La mayoría de bombas de la familia MATE expulsan un espectro relativamente amplio de compuestos tóxicos para la célula (181). Con el objetivo de indagar sobre la naturaleza del sustrato o sustratos de DinF, se determinó la concentración mínima inhibitoria de un panel de compuestos que se han usado en estudios similares (156, 184). Notablemente, no se encontraron diferencias para ninguno salvo para sales biliares, un agente oxidante e inductor de la respuesta SOS (185) que las bacterias que habitan el tracto digestivo deben soportar con frecuencia. De hecho, la eliminación de *dinF* confiere una desventaja competitiva en presencia de sales biliares, lo que apoya la idea de que confiere una protección contra varios estreses de naturaleza oxidante.

A tenor de los datos presentados anteriormente resulta tentador especular que DinF es una bomba de membrana encargada de mantener la homeostasis celular en períodos de estrés. Parece razonable suponer que, aparte de reparar el daño en el DNA, el sistema SOS disponga de las herramientas proteicas necesarias para sanear el interior celular, eliminando sustancias tóxicas (oxidantes o no). De ser así, se trataría de la primera bomba conocida que se regula en respuesta a daño en el DNA. Desafortunadamente, la naturaleza del sustrato o sustratos de DinF no fue revelada durante nuestro estudio. La hipótesis más razonable a la luz de los datos consiste en que DinF es capaz de expulsar algún intermediario oxidante. Quizás de un modo análogo, se ha descrito que las bombas TolC y AcrAB son capaces de expulsar compuestos conocidos como *redox cycling compounds* que desencadenan reacciones de oxidación. Además su expresión está regulada por el sistema sensible a estrés oxidativo SoxRS (182), lo que apoya la idea de que expulsar compuestos oxidantes puede suponer una estrategia válida para mantener unas condiciones redox propicias en el interior celular.

En cualquier caso, esta parte de la tesis supone la primera caracterización del gen SOS *dinF* en *E. coli*, en la que se demuestra que su sobreexpresión es capaz de reducir el estrés oxidativo, proteger frente a sales biliares e, incluso, atenuar la mutagénesis

producida por la deleción de *mutT*.

En conjunto, los dos trabajos a los que hace referencia lo expuesto anteriormente dan una idea general de cómo se regula la tasa de mutación de *E. coli* en respuesta a diferentes ambientes. Gracias al sistema SOS, la tasa de mutación es dinámica y depende de las circunstancias en las que se encuentre la bacteria, lo que pone de manifiesto que su ajuste, como el de todo parámetro biológico, está sujeto a la acción de la selección natural.

Siguiendo esta línea argumental, es de esperar que la magnitud de otros mecanismos evolutivos, como la recombinación, sean también objeto de la selección natural. Un indicio confirmatorio sería encontrar variabilidad de tasas de recombinación según el ambiente, así como organismos que presenten una diversidad de tasas de recombinación, condición *sine qua non* para la acción de la selección natural.

Sobre la recombinación en cepas naturales de *E. coli*

Con el fin de arrojar luz sobre estas y otras cuestiones, nos propusimos cuantificar la frecuencia de recombinantes en poblaciones naturales de *E. coli*. Para ello, en el tercer artículo, utilizamos un sistema basado en un plásmido que nos permite estimar el número de entrecruzamientos por célula viable en una población bacteriana. Mediante esta herramienta calculamos la frecuencia de recombinantes de una colección bien caracterizada (21, 158, 159) de 160 cepas naturales de *E. coli* (comensales y ExPEC).

Los resultados inducen a pensar que la tasa de recombinación es muy variable dentro de la especie, ya que encontramos cepas cuyas frecuencias se distribuyen a lo largo de varios órdenes de magnitud. Además esta variabilidad se manifiesta también en diferentes condiciones, como al crecer en orina, un medio al que muchas ExPEC deben enfrentarse para producir una infección. En este ambiente, que puede considerarse estresante por la alta salinidad y el estrés oxidativo (186), las cepas muestran una tendencia a incrementar la frecuencia de recombinación, comparando con la obtenida en LB. Sin

embargo, cabe destacar que algunas cepas presentan un descenso en la frecuencia de recombinación, lo cual podría implicar una buena adaptación previa a este medio. En cualquier caso, este resultado sugiere que la recombinación, al igual que la mutación, es variable según las condiciones y está sujeta a la acción de la selección natural.

Otro resultado que apoya esta idea proviene de la clasificación de las cepas según el origen de su aislamiento, lo que demuestra que los ExPEC presentan unos niveles de recombinación muy superiores a las cepas comensales. Tomando la mediana como estimador de tendencia central, esta diferencia está cerca de los dos logaritmos. No obstante, la distinción entre cepas ExPEC y comensales es siempre ambigua, dado que las cepas capaces de causar enfermedades extra-intestinales son patógenos facultativos que pertenecen a la microbiota normal de individuos sanos y sólo bajo ciertas circunstancias (como al alcanzar el tracto urinario, por ejemplo) desarrollan su carácter patógeno (187). Este hecho hace que la frontera entre una cepa comensal y una patógena no esté bien definida y sea más bien laxa. En nuestra colección, las cepas fueron clasificadas según la procedencia del aislado (de diversas infecciones o de heces de individuos sanos), por lo que existe cierta incertidumbre al respecto. Una distinción menos categórica y quizás más realista, consiste en cuantificar el número de determinantes de virulencia presentes en cada cepa. Se ha demostrado que existe una fuerte correlación positiva entre el número de genes de virulencia y la patogenicidad medida en un modelo de ratón (159), es decir, las cepas que poseen más determinantes de virulencia tienden a matar más ratones. La clasificación según este parámetro refuerza el principal hallazgo del nuestro trabajo; existe una correlación moderada, pero significativa, entre la frecuencia de recombinación y la cantidad de genes de virulencia presentes en cada cepa.

De un modo similar, un estudio genómico previo encontró evidencias de altas tasas de recombinación en cepas patógenas intestinales de *E. coli* (188). La concordancia entre ambos resultados, inferidos por técnicas muy diferentes (MLST; *multilocus sequence typing* y estimación directa basada en un plásmido), refuerza la idea de que la

patogenicidad en *E. coli* está directamente relacionada con altas tasas de recombinación. Esta asociación ha sido sugerida también para otros patógenos facultativos como *Moraxella catarrhalis* (189), un comensal del tracto respiratorio humano que ocasionalmente puede causar infecciones (189, 190).

Otras observaciones que podrían reforzar esta hipótesis se basan en un corolario de la recombinación: el tamaño del genoma. Se ha argumentado que un aumento de la recombinación y por tanto de una mayor probabilidad de adquisición de genes exógenos por HGT, puede tener como consecuencia un incremento en el tamaño del genoma. Siguiendo esta línea, aquellos ecotipos que presenten mayor frecuencia de recombinación tenderían a presentar como consecuencia un genoma mayor. De hecho, las cepas patógenas de *E. coli* suelen poseer un genoma más grande que las comensales (187) lo que podría interpretarse como un reflejo de las diferencias en recombinación demostradas durante el desarrollo de esta tesis.

Los datos presentados hasta este punto ayudan a consolidar la idea de que la recombinación y la virulencia están estrechamente relacionadas, al menos en *E. coli*. Sin embargo, los causas de dicho nexo no están en absoluto esclarecidas.

De hecho, la selección de un fenotipo hiperrecombinador por las cepas ExPEC puede tener, al menos, tres interpretaciones no necesariamente excluyentes entre sí (esquematizadas en la figura 2): La primera, relacionada con la hipótesis de la Reina Roja, estipula que las cepas patógenas, involucradas en una "carrera armamentística" con su hospedador, necesitan un contexto genómico muy dinámico, es decir, con mayor capacidad para adquirir y reordenar fragmentos génicos. Esto les permite escapar del sistema inmune e incluso obtener resistencia a antibióticos. Consecuentemente, el fenotipo hiperrecombinador sería coseleccionado con las ventajas adaptativas que produce, un mecanismo que recuerda a la selección de segundo orden que ha sido invocada para explicar la prevalencia de mutadores en poblaciones naturales (24).

Una segunda interpretación es que una maquinaria de recombinación más

eficiente podría significar una mayor capacidad para reparar con el daño en el DNA producido por el ambiente estresante al que tienen que enfrentarse las cepas patógenas. Por ejemplo, se ha demostrado que la orina, donde se desarrollan la mayoría de infecciones extra-intestinales, produce estrés oxidativo en cepas naturales (186), lo que puede ser causa de daño en el DNA (191). Bajo estas condiciones, las cepas con mayor tasa de recombinación pueden tener más probabilidades de sobrevivir, lo que explicaría la selección de cepas hiperrecombinadoras. Con respecto a esta hipótesis, conviene señalar que las cepas aisladas de infecciones de tracto urinario presentan las mayores tasas de recombinación de nuestra colección.

Las dos interpretaciones anteriores asumen que la selección de la hiperrecombinación es posterior (o simultánea) al desarrollo de la patogenicidad. En cambio, la tercera explicación supone que es una alta tasa de recombinación preexistente lo que ha facilitado la adquisición de genes de virulencia por cada cepa, acelerando la transición del comensalismo a la patogenicidad. Según esta idea, las cepas comensales con mayores tasas de recombinación serían más propensas a adquirir factores de virulencia y, por lo tanto, convertirse en patógenos. No obstante, esta interpretación se basa en que la adquisición de genes de virulencia debe representar alguna ventaja adaptativa, de modo que los pasos que llevan a un comensal a convertirse en un patógeno estén favorecidos evolutivamente.

A ese respecto, la hipótesis de la evolución casual (*coincidental evolution hypothesis*, en inglés) sugiere que los factores de virulencia son, en realidad, adaptaciones ventajosas en otros ambientes y que la patogenicidad es una consecuencia fortuita (192, 193). Esta teoría estipula que la virulencia no emerge de la coevolución entre el huésped y la bacteria, sino que surge por casualidad como subproducto de adaptaciones no relacionadas. Por ejemplo, se ha demostrado que determinados transportadores de hierro tradicionalmente considerados genes de virulencia, confieren resistencia a *E. coli* frente a la depredación por protozoos (194), por lo que no está claro cuál habría sido la

causa de su selección. Por lo tanto, cabe la posibilidad de que la hiperrecombinación se asocie a la virulencia, gracias a que aumenta la probabilidad de adquirir islas de patogenicidad que conferirían una ventaja selectiva mucho antes de dotar a la bacteria con la capacidad de producir una infección.

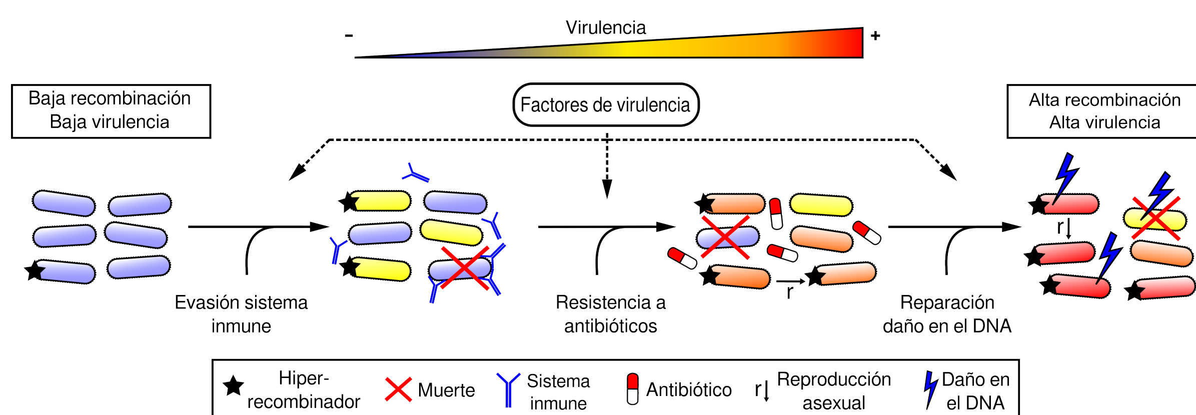

Figura 2. *Modelo que ilustra la posible importancia de la recombinación en la transición del comensalismo a la patogenicidad. La evasión del sistema inmune, la resistencia a tratamientos antibióticos o la capacidad para resistir el daño en el DNA, son algunos ejemplos que podrían coseleccionar una mayor frecuencia de recombinación (señalado en el diagrama como un incremento en la proporción de bacterias con alta tasa de recombinación). En el transcurso de este proceso se produce la adquisición gradual de factores de virulencia y la patogenicidad de la población aumenta (señalado con el cambio gradual de color en algunos individuos).*

Obviamente, estas hipótesis (salvo el modelo de reparación del DNA) asumen que la recombinación de fragmentos génicos en el genoma de la célula receptora es un paso clave para la HGT y, por tanto, para la adquisición de nuevas características. Esto parece ser cierto al menos con respecto a las islas de patogenicidad, pues tienden a localizarse en el cromosoma (195, 196), lo que sugiere un escenario en el que la recombinación resulta crucial para su integración y mantenimiento.

En cualquier caso, es probable que otros pasos de HGT, como la transferencia de DNA o su degradación por sistemas de restricción, muestren una variabilidad similar a la que se ha descrito para la mutación y la recombinación a lo largo de esta tesis.

Algunas evidencias apuntan en esta dirección. Por ejemplo, se ha demostrado que existe una gran diversidad en la frecuencia de conjugación entre cepas de *E. coli* (197). Sería interesante conocer si las diferencias en recombinación entre cepas patógenas y comensales que hemos observado en esta tesis tienen un reflejo en la frecuencia de conjugación. Adicionalmente, se ha descrito que factores ambientales pueden tener un papel clave en la modulación de HGT (198). Por ejemplo, el tracto gastrointestinal es un ecosistema que resulta especialmente propicio para la transferencia génica, ya que proporciona un flujo continúo de nutrientes a los microorganismos que lo habitan, lo que permite que mantengan un metabolismo activo y alcancen una alta densidad de población (198). Además, la enorme heterogeneidad de especies que lo habitan favorece el denominado efecto amplificador, mediante el cuál se puede acelerar la frecuencia de HGT (197).

En la última parte de esta discusión, sin embargo, se analizan las implicaciones de un sistema de transferencia génica cuya regulación escapa a la selección natural, pues no está mediada por componentes biológicos.

Sobre la transferencia genética abiótica mediada por sepiolita

Previamente al desarrollo de esta tesis se había descrito que la sepiolita y otros materiales arcillosos son capaces de introducir plásmidos en bacterias si se aplica una fuerza de fricción (44). El proceso se basa en que estos materiales son capaces de adsorber el DNA plasmídico libre, generando complejos punzantes que son capaces de penetrar la membrana celular gracias a la fricción. Una vez dentro, ácidos nucleicos intracelulares compiten por la unión a la arcilla, lo que libera el DNA plasmídico en el citoplasma, transformando la bacteria (199). Esta propiedad de los materiales arcillosos se ha propuesto como un sistema poco laborioso para la transformación rutinaria de laboratorio y se ha demostrado funcional en múltiples especies bacterianas (45, 46).

El objetivo del cuarto artículo de esta tesis fue comprobar si la sepiolita, dadas las

propiedades anteriormente mencionadas, era capaz de mediar la transferencia horizontal de DNA, directamente de una célula donadora a una receptora. Los resultados demuestran que los plásmidos se transfieren fácilmente, incluso entre diferentes especies bacterianas, lo que inmediatamente sugiere un protocolo de laboratorio que evita la extracción de DNA plasmídico y la preparación de células competentes para la transformación en condiciones rutinarias de laboratorio.

Sin embargo, esta transferencia mediada por arcillas podría tener implicaciones que van más allá de la rutina del laboratorio y que han sido consideradas en el quinto artículo de la presente tesis. La sepiolita es un suplemento alimentario muy extendido en la industria pecuaria (200, 201), la cual se ha considerado un gran reservorio de resistencia a antibióticos (202). La combinación de las arcillas presentes en el pienso animal junto con las enormes densidades microbianas en el tracto digestivo del ganado y la fuerza de fricción producida por la peristalsis del intestino podrían proporcionar los ingredientes necesarios para que se dé la transferencia mediada por sepiolita. Adicionalmente, las ingentes cantidades de antibióticos que se administran rutinariamente al ganado o podrían ejercer la presión selectiva necesaria para el mantenimiento y diseminación de genes de resistencia.

En el tracto digestivo del ganado se dan unas condiciones físico químicas (temperatura, pH, salinidad y presión) que están dentro del rango de lo que se ha reportado como óptimo para la transformación mediada por sepiolita. Esto nos lleva a hipotetizar que la sepiolita podría estar acelerando la tasa de transferencia de factores de resistencia o virulencia en el tracto digestivo de animales de granja, por lo que su uso como suplemento alimentario debería ser reevaluado por las autoridades.

Por otra parte, el descubrimiento de que una sustancia abiótica es capaz de propiciar la transferencia directa de material genético es quizás una de las aportaciones más originales de este trabajo. Además, este mecanismo presenta algunas ventajas desde el punto de vista de la eficacia de la transferencia horizontal. Por ejemplo, con respecto a

la conjugación, la transformación mediada por sepiolita no requiere coexistencia espacial ni temporal ya que el DNA puede ser adsorbido y retenido por la sepiolita (199). De hecho, la sepiolita presenta la capacidad de ofrecer cierta protección frente a degradación por nucleasas (203), lo que representa una ventaja con respecto a la transformación natural. En este sentido comparte algunas de las características de la transducción, con la salvedad de que, aparentemente, carece del estrecho espectro de infección que presentan los fagos, lo que amplia el rango de especies susceptibles de adquirir DNA mediante este fenómeno.

"Scientific discovery and scientific knowledge have been achieved only by those who have gone in pursuit of it without any practical purpose whatsoever in view"

Max Plank
Where is science going?

Capítulo VI
Conclusiones

1.- Un tratamiento corto con concentraciones en torno a la mínima inhibitoria provoca un incremento consistente en la mutagénesis en ocho de los trece antibióticos ensayados coincidente, en la mayoría de los casos, con la inducción del sistema SOS.

2.- La eliminación de *recA* produce un moderado aumento en la sensibilidad a varios antimicrobianos y suprime el aumento de la mutagénesis producida por el tratamiento antibiótico, salvo en el caso de la ceftazidima. Por lo tanto, la inhibición de RecA presenta un enorme potencial terapéutico, pues aumenta la efectividad del tratamiento con ciertos antibióticos al tiempo que inhibe la aparición de resistencia por mutación.

3.- La sobreexpresión del gen SOS *dinF* de *E. coli* protege frente a peróxido de hidrógeno, reduce el estrés oxidativo intracelular y la carbonilación proteica, confiere una ventaja adaptativa en presencia de sales biliares y es capaz de atenuar la mutagénesis producida por la deleción de *mutT*.

4.- El plásmido pRhomo es capaz de medir eventos de recombinación, en la mayoría de los casos RecA-dependientes, en cepas naturales de *E. coli*. Las frecuencias obtenidas muestran una buena correlación con las calculadas mediante una construcción cromosómica en un subgrupo de cepas y las variaciones en el número de copias del plásmido entre cada cepa no explican los resultados de recombinación.

5.- La frecuencia de recombinación en cepas naturales de *E. coli* es muy diversa y variable según el ambiente. Los patógenos extra-intestinales presentan frecuencias más altas que sus congéneres comensales, especialmente las cepas aisladas de infecciones de tracto urinario. Además, la correlación positiva entre virulencia y recombinación refuerza la idea de que patogenicidad y recombinación están estrechamente relacionadas.

6.- La sepiolita, unida a fuerzas de fricción, es capaz de mediar la transferencia directa de plásmidos entre diferentes especies bacterianas. Además, el tracto digestivo del ganado podría representar el escenario óptimo para que dicha transferencia tuviera lugar, ya que la sepiolita se usa como aditivo alimentario, se encuentran fuerzas de fricción y existen altas densidades bacterianas.

Capítulo VII
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Capítulo VIII

Anexos

Anexo I. Artículos publicados durante el desarrollo de esta tesis

1. *Can clays in livestock feed promote antibiotic resistance and virulence in pathogenic bacteria?*

Alexandro Rodríguez-Rojas, Jerónimo Rodríguez-Beltrán, José Ramón Valverde y Jesús Blázquez. *Antibiotics.* Julio de 2015.

2. *High recombinant frequency in extraintestinal pathogenic Escherichia coli strains.*

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Anexo II. Observaciones sobre la escritura de la presente tesis

Habrá notado el lector que a lo largo de la presente tesis este doctorando ha optado por incluir todas las abreviaturas y acrónimos en inglés. Así, quizás haya resultado chocante leer DNA en vez de ADN en un manuscrito redactado en castellano. El motivo de esta decisión no es otro que el de ser coherente con otras abreviaturas que se han preferido escribir en inglés por lo reconocido de su grafía. Por ejemplo, HGT o ROS son abreviaturas conocidas ampliamente en el mundo de la microbiología (e incluso fuera de él), mientras que su transcripción en español (THG y SRO) es, a buen seguro, más confusa. Por coherencia, esta decisión se ha mantenido para todas las abreviaturas, incluso aquellas que sí poseen una versión reconocible en castellano como es el caso del DNA. Pido disculpas por esta licencia y por el hecho de que el lector no esté, necesariamente, de acuerdo.

Anexo III. Material suplementario

Supplementary material for:

High recombinant frequency in extra-intestinal pathogenic *Escherichia coli* strains

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Supplementary Methods

***Construction of plasmid* pRhomo**

To construct plasmid pRhomo (supplementary figure 1), a 1223 bp fragment, containing the promoter region and the first 939 bp of the 5' region of the *tetA* gene (denominated *tetA*-5'), was obtained by PCR amplification with primers 5'-TetA-SacXhoF and 5'-TetA-KpnIR from plasmid pBBR1MCS3 (Kovach, et al. 1995) and cloned in pGEM-T Easy, to give pGEM-TEasy-*tetA*-5'. A 975 bp fragment, containing the last 864 bp of the 3' fragment of the *tetA* gene (denominated *tetA*-3'), was obtained from the same plasmid by PCR amplification with primers 3'-TetA-XbaF and 3'-TetA-PstXhoR and cloned in pGEM-T to give pGEM-T-*tetA*-3'.

The *aacC1* gen, conferring gentamicin resistance (GmR), was PCR amplified from plasmid pUCGmlox with primers Gm-SacR and Gm-SacF (Quenee, et al. 2005) and cloned in pGEM-T Easy. The *aacC1* gene was extracted by restriction with *Not*I and cloned into pGEM-TEasy-*tetA*-3', previously linearized with the same restriction enzyme, rendering plasmid pGEM-TEasy-Gm-*tetA*-3'. This plasmid was digested with *Nco*I and their cohesive ends were blunted with Mung Bean nuclease. This product was digested with *Nsi*I, rendering the fragment Gm-*tetA*-3' with blunt and *Nsi*I ends. This fragment was purified and cloned in pGEM-T-*tetA*-5' digested with *Nde*I (further blunted with Mung Bean nuclease) and *Nsi*I, redering plasmid pRhomo. Cloning was verified by restriction digestion and sequencing.

Therefore, plasmid pRhomo bears the tetracycline resistance (TetR) gene *tetA* truncated in two non-functional fragments (*tetA* 5' and *tetA* 3') that share 627 bp of 100% homology (dashed region in supplementary figure 1) which do not confer tetracycline resistance by themselves. If a recombination event occurs between these homologous regions, the *tetA* gene is restored, conferring resistance to tetracycline.

Construction of K-12 strains with chromosomal single copy insertions for recombinant frequency measurements

For the construction of the strain MG1655 harboring the single-copy insertion of the *tetA1-kan-tetA2* construct in the chromosome, plasmid pTetKanTet (supplementary figure 3A) was constructed. Sequential PCR and ligation reactions were used to obtain a DNA fragment that contains the promoter region and the first 437 bp of the *tetA* tetracycline resistance gene from pBR322 (Bolivar, et al. 1977), the complete functional *kan* kanamycin

resistance gene from pKD4 (Datsenko and Wanner 2000), and the complete *tetA* gene with the exception of the promoter region and the start codon (Bolivar, et al. 1977). The 2 *tetA* fragments share a 390 bp-long homology. Roche's long range PCR fragment expending kit was used to amplify this *tetA1-kan-tetA2* fragment with primers XhoTetUp and NotTetDn according to manufacturer's instructions. This PCR introduces an *Xho*I and a *Not*I restriction site at the ends of the amplicons. These restriction sites were used to clone the PCR product into pGRG36 (McKenzie and Craig 2006) multicloning site. Plasmid pGRG36 contains the Tn*7* transposing machinery (Peters and Craig 2001) and harbors a temperature-sensitive origin of replication, a conjugative origin and an ampicillin resistance *bla* gene (AmpR). The ligation product was electroporated into the D10HB *E. coli* strain and transformants were selected on ampicillin and kanamycin at 30°C. Integrity of the *tetA1-kan-tetA2* fragment was checked by sequencing.

When this plasmid is introduced into an *E. coli* strain (by transformation or conjugation), the Tn*7* transposase machinery drives the chromosomal single-copy insertion of the *tetA1-kan-tetA2* construction to the *att*Tn*7* site, which is located at the 3' side of *glmS* gene (encoding the glucosamine 6-phosphatase) (Peters and Craig 2001) (supplementary figure 3B).

To obtain strains MG1655 *att*Tn*7::tetA1-kan-tetA2* and MG1655 *recA att*Tn*7::tetA1-kan-tetA2*, plasmid pTetKanTet was introduced into W7249 strain, a diaminopimelate (DAP)-dependent strain of *E. coli* that contains a chromosomal insertion of the conjugation machinery of RP4 plasmids (Babic, et al. 2008) by electroporation. Cells were incubated at 30°C for 2h before being plated on LB agar plates containing ampicillin (50 µg/ml) and DAP (300 µM) at 30 °C. One transformant was selected.

A mixture of 50 µl of an overnight culture of MG1655 (grown in LB at 37 °C) and of 100 µl of an overnight culture of W7249 (pTetKanTet) (grown in LB + DAP) was plated over an LB agar + DAP plate and incubated overnight at 30°C. The following day, a sample was streaked on an LB-agar (containing Amp and Kan) plate at 30°C. This step gets rids of the W7249 donor strain (DAP-auxotrophy). The following day, several clones were separately inoculated in LB and grown overnight at 30°C and then plated on LB-agar at 42°C. This step gets rid of pTetKanTet, which has a temperature-sensitive origin of replication. On the next day, several clones were streaked in parallel on LB-agar + Amp and on LB-agar + Kan at 42°C. One clone that was sensitive to ampicillin and resistant to kanamycin was selected for further confirmation of its chromosomal integration of the *tet1-kan-tet2* fragment. The exact same

procedure was performed on K-12 MG1655 *recA*.

The chromosomal insertion and the loss of the pTetKanTet plasmid were checked by two PCRs with primers, designed to amplify the junction of the *tet1-kan-tet2* fragment and either the chromosome or pTetKanTet. Chromosomal insertion in single copy was verified by Southern blot and sequencing (data not shown).

*Generation of Δ*recA::kan *mutants in natural strains*

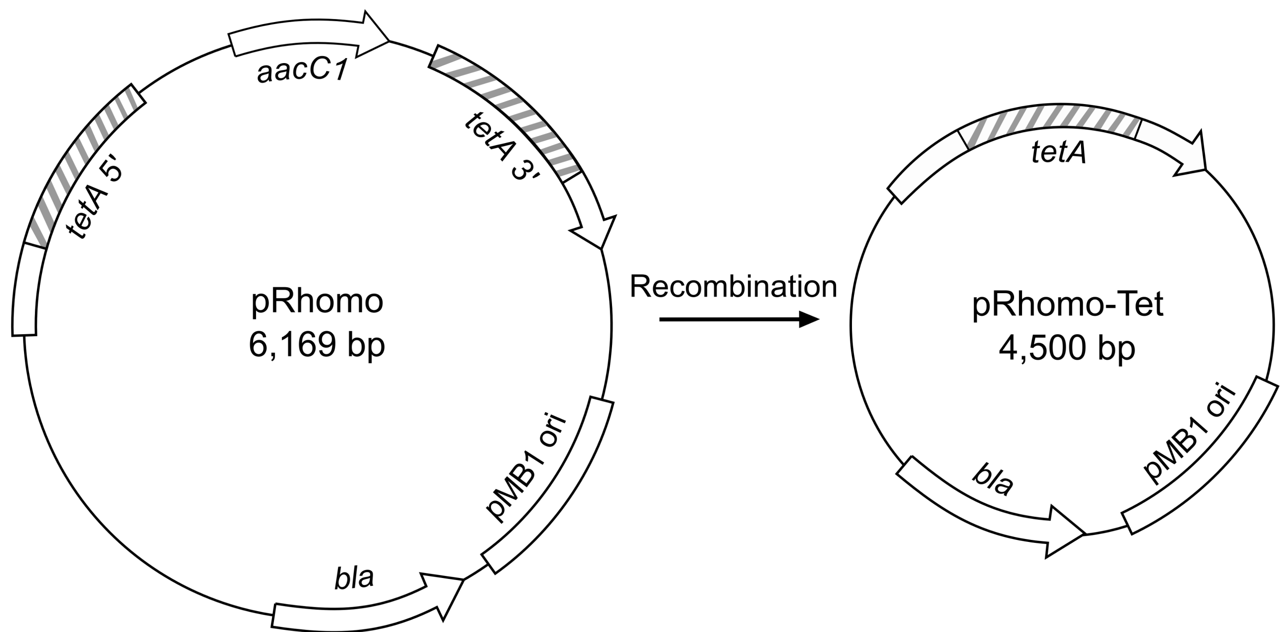
To delete the *recA* gene of a subset of strains of the collection, a modification of the λ Red recombinant method was used (Datsenko and Wanner 2000). The oligonucleotides recAF and recAR (supplementary table 2) were used to amplify the kanamycin resistance gene with ~300bp extensions corresponding to the flanking regions of the *recA* gene, using the *ΔrecA::kan* mutant from the KEIO collection (Baba, et al. 2006) as template. Using the KEIO mutant as a template, we were able to obtain longer homologous regions than normally obtained by using the pKD3/pKD4 plasmids. This is convenient, as the sequences flanking the *recA* gene in most of the strains in our collection are unknown and could present different levels of polymorphism. Enlargement of the homologous regions enhances the chances of λ Red mediated recombination to produce the desired deletion. 100-500ng of the amplified *ΔrecA::kan* template were electroporated into cells carrying pKOBEG plasmid (Chaveroche, et al. 2000). Induction of λ Red genes was achieved by using 10mM of arabinose. Deletion of *recA* was verified by PCR using primers recAoutF/K1 and recAoutR/K2.

Relative plasmid copy number estimation by qPCR.

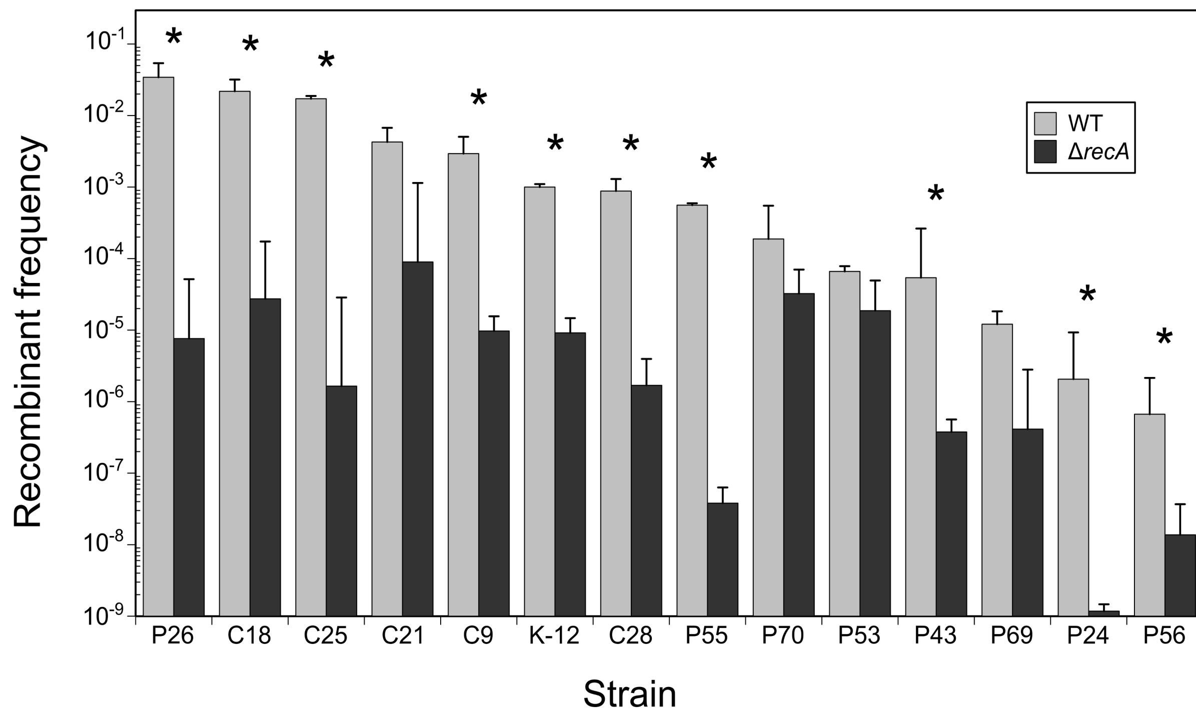
The relative plasmid copy number was estimated by quantitative PCR (qPCR) for three independent transformants from 28 randomly chosen strains (14 commensals and 14 pathogens) as previously described (Skulj, et al. 2008), using ME12 (pRhomo) as reference. Briefly, a primer pair was designed to amplify a chromosomal DNA probe located in the *dnaE* gene (supplementary table 2). This probe shares 100% homology in all sequenced *E. coli* strains to date, as determined by BLAST search. A plasmidic probe, consisting of two oligonucleotides hybridizing in a non-duplicated region at the beginning of the *tetA* gene, was also designed. All primers were designed by using Primer3Plus software (Untergasser, et al. 2007). Reactions that amplified both probes showed similar efficiencies (99% and 97%) and therefore the ΔΔCt method was validated and chosen for data analysis (Livak and Schmittgen 2001). Strains were grown in LB broth with ampicillin and gentamicin overnight, centrifuged,

resuspended in MQ water, boiled for 20 min at 95ºC and immediately stored at -80ºC. Before addition to the reaction mix, samples were thawed and centrifuged to spin down cellular debris. The supernatant was diluted 1:100 and 2 μl were added as template per reaction. SYBR Select Master Mix (Applied Biosystems) was used and reactions of 15 μl were prepared as recommended by the manufacturer with an oligonucleotide final concentration of 300nM. All reactions were carried out in a ViiA7 Real-Time PCR System (Applied Biosystems). ViiA7 software was used for data analysis.

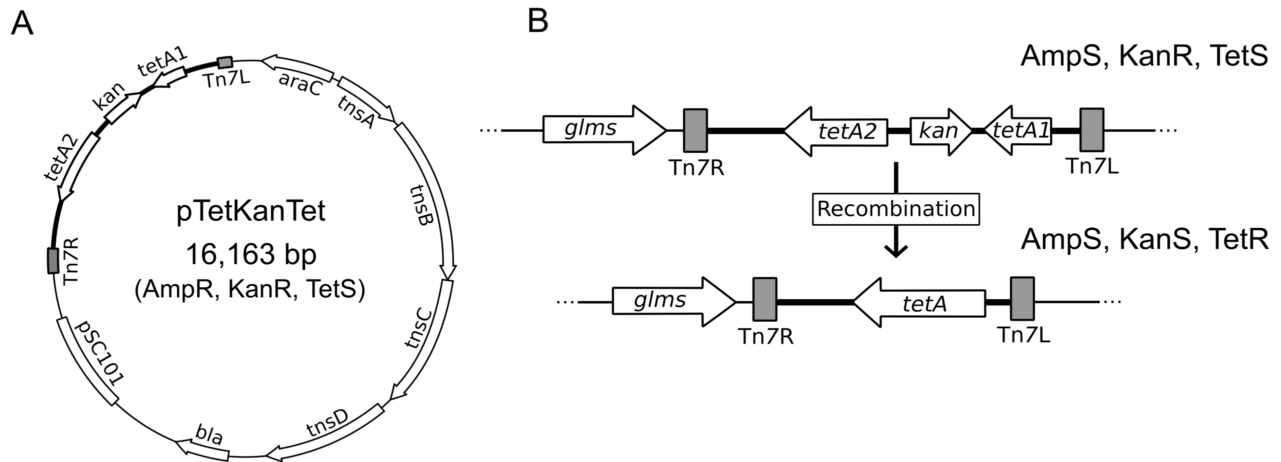
Supplementary Figures



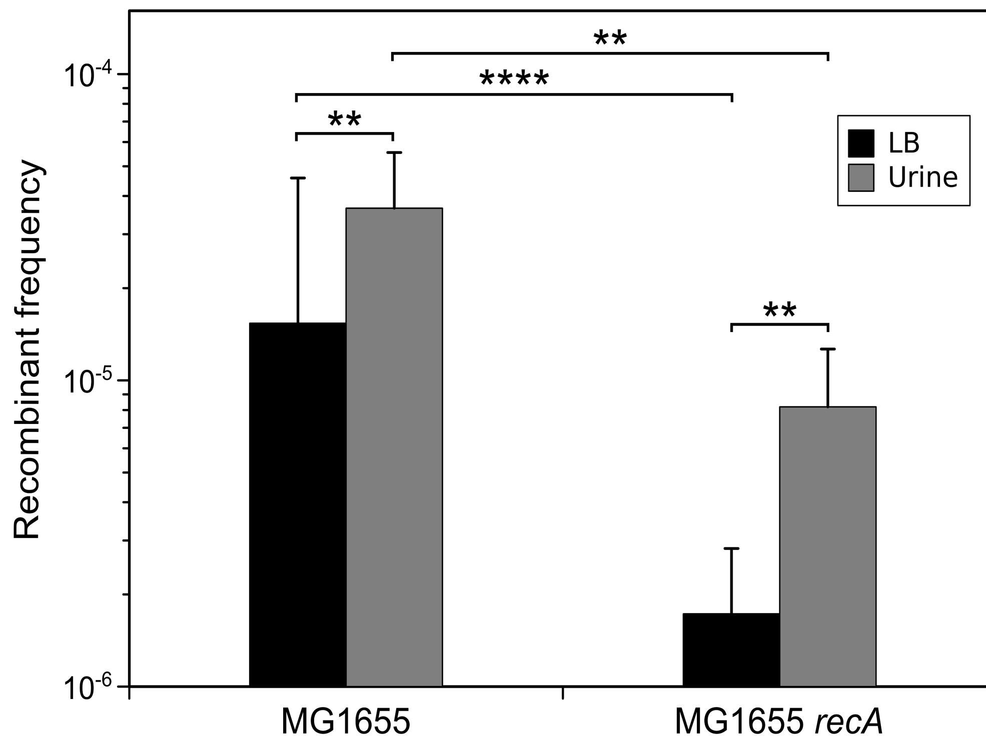
Supplementary Figure 1. Map of plasmid pRhomo before and after recombination. *tetA*5' and *tet*A3' are non-functional *tetA* alleles with C-terminal or N-terminal deletion, respectively, in pRhomo (AmpR GmR). The two alleles share an overlapping 100% homologous DNA region of 627 bp (dashed boxes) and are separated by a 1,042 bp fragment containing the *aacC1* (GmR). One recombination event between the two *tetA* alleles restores a functional *tetA* gene, originating plasmid pRhomo-Tet (AmpR TetR).



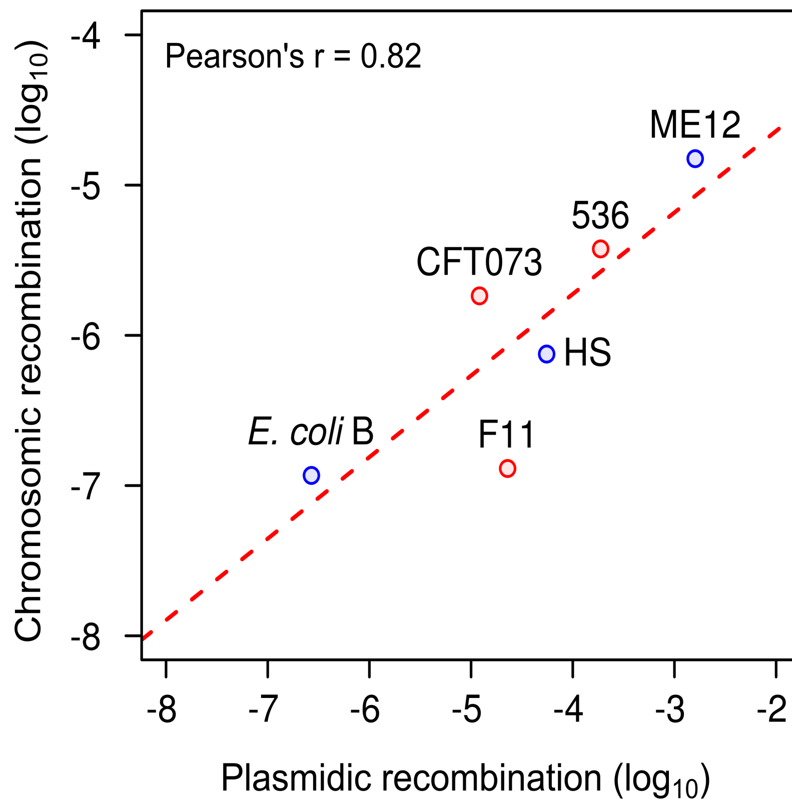
Supplementary Figure 2. Effect of *recA* deletion on TetR recombinant frequencies. The *recA* gene was deleted in 13 strains of the collection as well as in the K-12 laboratory strain. Recombinant frequencies (median ± s.e.m.) for the Δ*recA* mutants (black bars) and the parental strains (grey bars) are shown. Strain names begin with either 'P' or 'C' indicating that the strain is a pathogen or a commensal, respectively. The asterisks denote significant differences (t-test for log10 transformed values; $p<0.05$). To facilitate understanding, strains appear ordered by recombinant frequency of the *recA*-proficient parental strains.



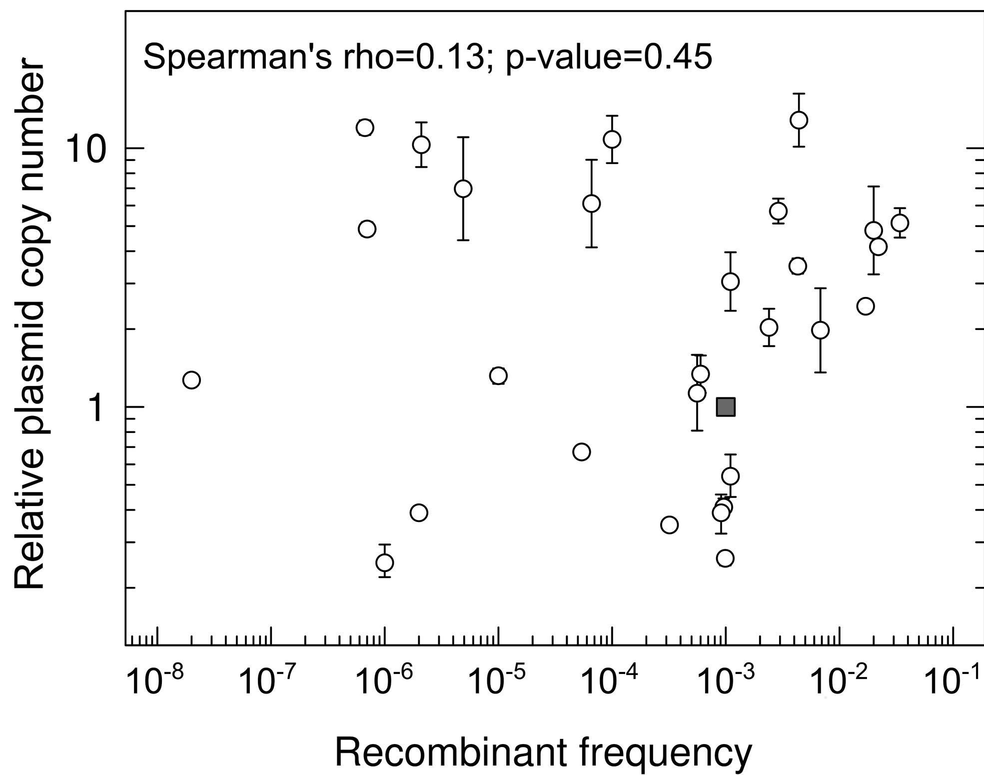
Supplementary Figure 3. Construction of the MG1655 strains harboring the chromosomal *att*Tn*7::tetA1-kan-tetA2* insertions. (A), Map of plasmid pTetKanTet, conferring resistance to ampicillin (AmpR) and kanamycin (KanR), used to construct the single copy insertions in strains MG1655 *att*Tn*7::tetA1-kan-tetA2* and MG1655 *recA att*Tn*7::tetA1-kan-tetA2.* Grey boxes in pTetKanTet indicate the Tn7R and Tn7L sequences that mediate the insertion of the *tetA1-kan-tetA2* construct. (B), Two overlapping non-functional segments of the *tetA* gene (*tetA1* and *tetA2*), sharing an homology of 100% and interspaced by a functional kanamycin resistance gene (*kan*), are inserted in the attTn*7* site of the *E. coli* chromosome, close to the *glmS* gene. When a recombination event occurs involving *tetA1* and *tetA2*, tetracycline resistance (TetR) is restored and kanamycin resistance is lost (KanS).



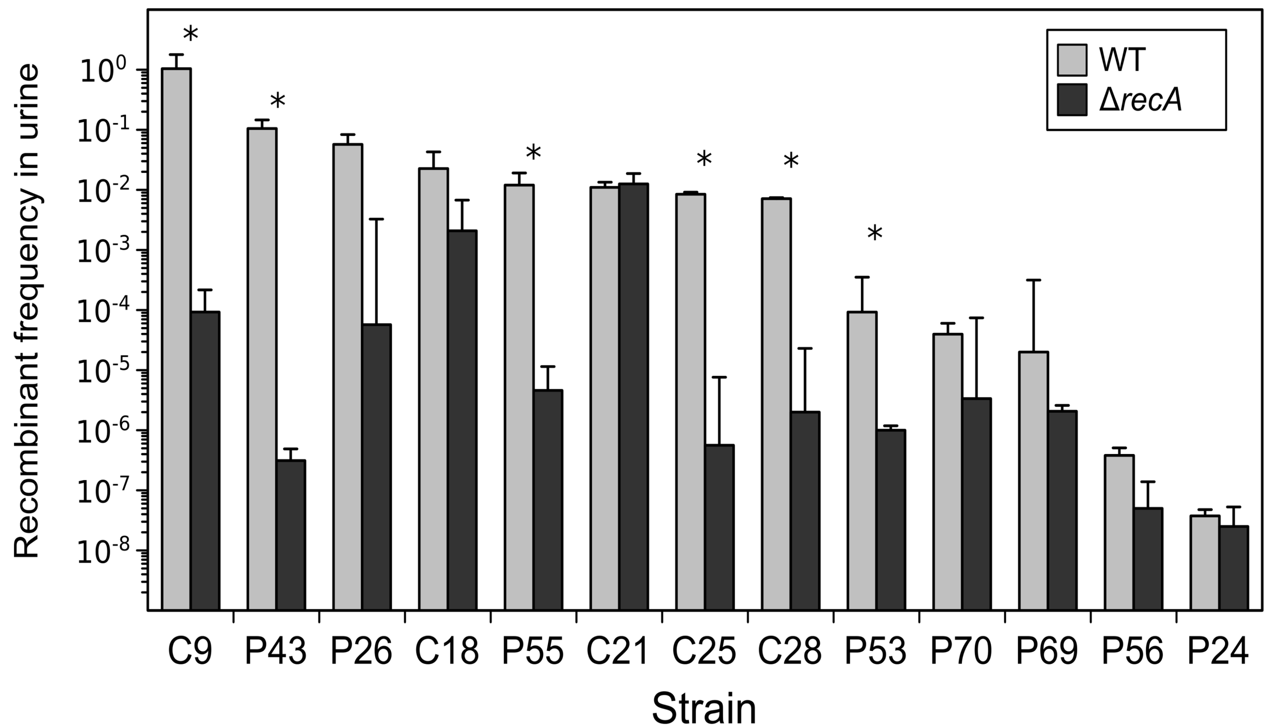
Supplementary Figure 4. Recombinant frequency in LB (black bars) and urine (grey bars) of *E. coli* K-12 strains MG1655 *att*Tn*7::tetA1-kan-tetA2* and its *recA*-derivative (MG1655 *recA att*Tn*7::tetA1-kan-tetA2*). Lines over the graph indicate significant differences (**: p-value <0.01; ****: p-value <0.0001; Mann-Withney U test).



Supplementary Figure 5. Comparison of recombinant frequencies of strains *E. coli* B, F11, HS, 536, CFT073 (strains C89, C90, P17, P123, P124 in supplementary table 1) and the K-12 strain ME12, harbouring either the plasmidic or the chromosomal system. A strong linear correlation between log transformed recombinant frequencies can be observed with both systems (Pearson's r=0.82, p-value=0.04). The dashed line represent the best linear fit for the data.



Supplementary Figure 6. Relative plasmid copy number and recombinant frequency are not correlated. The mean relative copy number of pRhomo is plotted against the recombinant frequency for each strain. Laboratory strain ME12 is represented as a grey square for reference. Error bars extend from minimal to maximum values of three replicates. No correlation was found (n=29, Spearman's rho=0.13; p-value=0.45), indicating that, although there is variability among strains, the copy number differences do not explain the recombinant frequencies observed.



Supplementary Figure 7. Effect of RecA on TetR recombinant frequencies in urine. The *recA* gene was deleted in 13 strains of the collection. Recombinant frequencies in urine (median ± s.e.m.) for the Δ*recA* mutants (black bars) and their *recA*-proficient parental strains (grey bars) are shown. Strain names begin with either 'P' or 'C' indicating that the strain is a pathogen or a commensal, respectively. The asterisks denote significant differences (t-test for log10 transformed values; $p<0.05$). To facilitate understanding, strains appear ordered by recombinant frequency of the *recA*-proficient parental strains.

Supplementary tables.

Supplementary Table 1. Strains and data used in this work are shown as a separate .xls file.

Supplementary Table 2. Oligonucleotides used in this study. Restriction sequences are in bold.

Oligonucleotide	Sequence 5'→3'	Restriction	Use
5'-TetA-SacXhoF	GCC**GAGCTCCTCGAG**GCCGTGGGCGAAAAGCTGCTG	*SacI/XhoI*	Amplification fragment 5' *tetA*.
5'-TetA-KpnIR	GCC**GGTACC**GAGAAGAATCATAATGGGGAA	*KpnI*	Amplification fragment 5' *tetA*
3'-TetA-XbaF	TAC**TCTAGA**CGCATCGTGGCCGGCATCACC	*XbaI*	Amplification fragment 3' *tetA*
3'-TetA-PstXhoR	CAT**CTGCAGCTCGAG**GCAAGAATTGATTGGCTCCAA	*PstI/XhoI*	Amplification fragment 3' *tetA*
Gm-SacS	TA**GAGCTC**TTCAGCTGTACAATTGG	*SacI*	Amplification *aacC1*, including promotor, from plasmid pUCGm*lox*
Gm-SacF	ATGT**GAGCTC**ACCGGTTAACACGCG	*SacI*	Amplification *aacC1*, including promotor, from plasmid pUCGm*lox*
XhoTetUp	AA**CTCGAG**CTGAAGTCAGCCCCATACGA	*XhoI*	Cloning of the TetKanTet fragment into pGRG36
NotTetDn	CCGCGGCCGCCTCGCGTATCGGTGATTCAT	*NotI*	Cloning of the TetKanTet fragment into pGRG36
DnaE qPCR F	GGCAAATTGATGACCAGCTT	-	Plasmid copy number estimation by qPCR
DnaE qPCR R	GCATCCGCCCTCTGATAGTA	-	Plasmid copy number estimation by qPCR
TetA qPCR F	ATGCGCTCATCGTCATCCTC	-	Plasmid copy number estimation by qPCR
TetA qPCR R	GGACGATATCCCGCAAGAGG	-	Plasmid copy number estimation by qPCR
*recA*F	ATAGGCGCACTGAAAAGCGG	-	Generation of *recA*::kan mutants
*recA*R	CAACAAATCGGCTGTCATCGAG	-	Generation of *recA*::kan mutants
k1	CAGTCATAGCCGAATAGCCT	-	Checking *recA*::kan mutants
k2	CGGTGCCCTGAATGAACTGC	-	Checking *recA*::kan mutants
*recA*outF	GGTAGCTCCGCCTGGTTTC	-	Checking *recA*::kan mutants
*recA*outR	CTGTCGCTTCGCGGGAAATA	-	Checking *recA*::kan mutants

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