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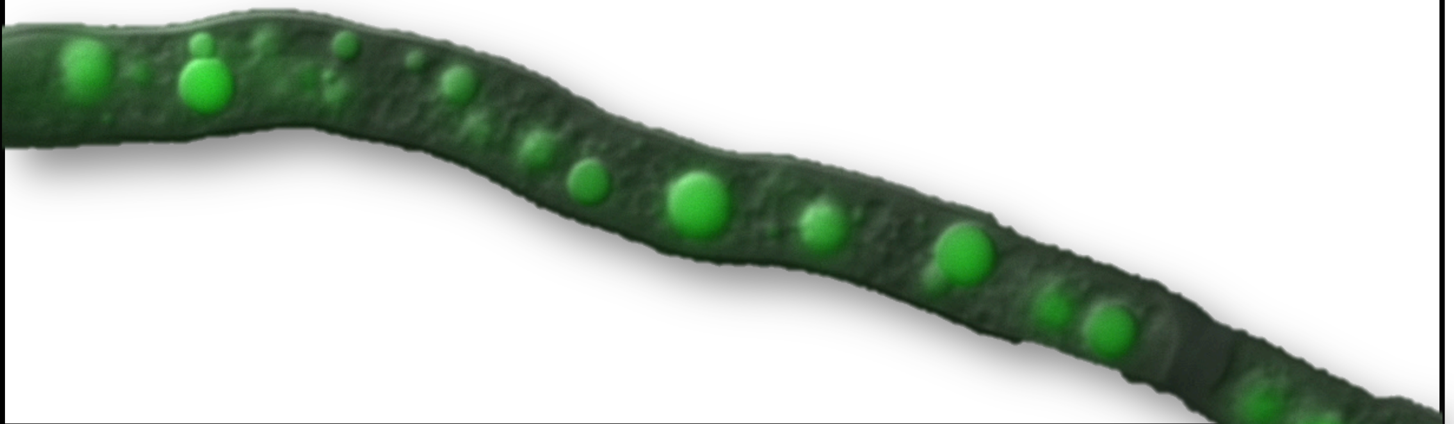
Departamento de Microbiología y Genética

Systems Metabolic Engineering in the industrial fungus *Ashbya gossypii*: boosting production of riboflavin, lipids and nucleosides

Ingeniería Metabólica de Sistemas en el hongo industrial *Ashbya gosypii*: impulsando la producción de riboflavina, lípidos y nucleósidos

Rodrigo Ledesma-Amaro
Tesis Doctoral
2014



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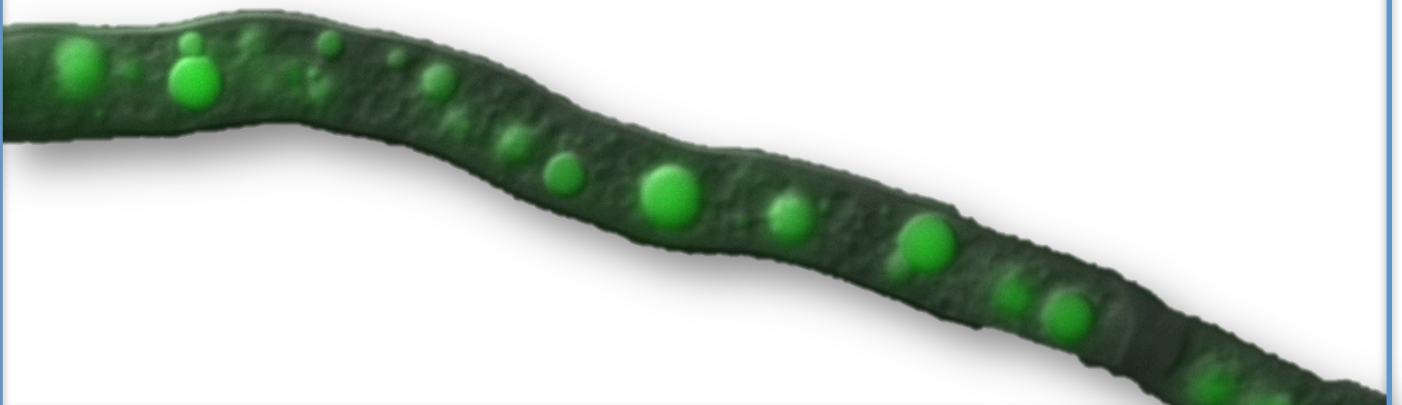

“Pleasure to me is wonder—the unexplored, the unexpected, the thing that is hidden and the changeless thing that lurks behind superficial mutability.”

H.P. Lovecraft

La presente memoria titulada "Systems Metabolic Engineering in the industrial fungus *Ashbya gossypii*: boosting production of riboflavin, lipids and nucleosides", elaborada por el licenciado e ingeniero Rodrigo Ledesma Amaro y que constituye su Tesis Doctoral para optar al grado de Doctor en Biotecnología con Mención Internacional, ha sido redactada en el formato de compendio de artículos originales de investigación publicados en revistas científicas de prestigio internacional e indexadas en la edición científica del *Journal Citation Reports*.

Y para que así conste se recogen a continuación los tres artículos originales de investigación requeridos, su título, autores y afiliación de los mismos, junto con la referencia completa de la revista científica donde fueron publicados:

"Genome Scale Metabolic Modeling of the riboflavin overproducer *Ashbya gossypii*"

Rodrigo Ledesma-Amaro[1], Eduard J. Kerkhoven[2], José Luis Revuelta[1], Jens Nielsen[2]

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Biotechnolgy and Bioengineering, volumen 111, número 6. páginas 1191-9 Junio 2014. DOI: 10.1002/bit.25167

"Strain design of *Ashbya gossypii* for single-cell oil production"

Rodrigo Ledesma-Amaro[1], María de los Ángeles Santos[1], Alberto Jiménez[1], José Luis Revuelta[1]

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Applied Environmental Microbiology, volumen 80, número 4, páginas 1237-44

DOI: 10.1128/AEM.03560-13

“Tuning single-cell oil production in *Ashbya gossypii* by engineering the elongation and desaturation systems”

Rodrigo Ledesma-Amaro[1], María de los Ángeles Santos[1], Alberto Jiménez[1], José Luis Revuelta[1]

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Biotechnolgy and Bioengineering, 25 de Marzo del 2014 (Publicado en linea).
DOI: 10.1002/bit.25245

Otros artículos y manuscritos adicionales incluidos en esta tesis son:

“Biotechnological production of feed nucleotides by microbial strain improvement”

Rodrigo Ledesma-Amaro[1], Alberto Jiménez[1], María de los Ángeles Santos[1], José Luis Revuelta[1]

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Process Biochemistry, volumen 48, número 9, páginas 1263-70, Septiembre 2013
DOI:10.1016/j.procbio.2013.06.025

“Microbial production of vitamins”

Rodrigo Ledesma-Amaro[1], María de los Ángeles Santos[1], Alberto Jiménez[1], José Luis Revuelta[1]

[1]Departamento de Microbiología y Genética, Metabolic engineering Group, University of Salamanca, Salamanca, España.

Microbial production of food ingredients, enzymes and nutraceuticals (Book) Woodhead Publishing Limited. 2013 ISBN 987-0-85709-354-1

“Fatty acid production in Eremothecium”

Patente: Revuelta JL[1], Jiménez A[1], Ledesma-Amaro R[1].

[1]Departamento de Microbiología y Genética, Metabolic engineering Group, University of Salamanca, Salamanca, España.

European Patent: EP-Patentanmeldung Nr. 13 190 151.4; BASF-Az.: PF75879 (INV0075879).

“Engineering of riboflaving overproduction in *A. gossypii*”

Ledesma-Amaro R[1], Serrano-Amatriain C[1], Jiménez A[1], Revuelta JL[1]

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(Manuscrito en preparación)

"Synthetic biology and metabolic engineering to produce polyunsaturated fatty acids in the industrial fungus *Ashbya gossypii*"

Ledesma-Amaro R[1], Lozano-Martínez P[1], Revuelta JL[1].

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(Manuscrito en preparación)

"Increased production of inosine and guanosine through metabolic engineering of the purine pathway in *Ashbya gossypii"*

Ledesma-Amaro R[1], Buey RM[1], Revuelta JL[1].

[1]Departamento de Microbiología y Genética, Metabolic engineering Group, University of Salamanca, Salamanca, España.

(Manuscrito en preparación)

"Increased riboflavin production by manipulation of inosine 5´-monophosphate dehydrogenase in *Ashbya gossypii"*

Buey RM[1], Ledesma-Amaro R[1], Balsera M[2], Pereda JM[3], Revuelta JL[1].

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(Manuscrito en preparación)

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DR. PEDRO F. MATEOS GONZÁLEZ, PROFESOR TITULAR DE LA UNIVERSIDAD DE SALMANACA Y PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO

CERTIFICA:

Que la memoria titulada "Systems Metabolic Engineering in the industrial fungus *Ashbya gossypii*: boosting production of riboflavin, lipids and nucleosides", presentada por el licenciado e ingeniero Rodrigo Ledesma Amaro, ha sido realizada bajo la dirección del Dr. José Luis Revuelta Doval, en el departamento de Microbiología y Genética de la Universidad de Salamanca.

Y para autorizar su presentación como tesis internacional en formato de compendio de artículos científicos y evaluación por el tribunal correspondiente, expide el presente certificado en Salamanca, a 11 de Junio de 2014.

Dr. Pedro F. Mateos González

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DR. JOSÉ LUIS REVUELTA DOVAL, CATEDRÁTICO DE GENÉTICA DEL DEPARTAMENTO DE MICROBIOLOGÍA Y GENÉTICA LA UNIVERSIDAD DE SALAMANCA,

CERTIFICA:

Que el licenciado e ingeniero Rodrigo Ledesma Amaro ha realizado el trabajo titulado "Systems Metabolic Engineering in the industrial fungus *Ashbya gossypii*: boosting production of riboflavin, lipids and nucleosides", bajo mi dirección en el departamento de Microbiología y Genética de la Universidad de Salamanca, para optar al grado de doctor en Biotecnología con Mención de Doctorado Internacional.

Que autorizo a la presentación de dicho trabajo en la modalidad de compendio de artículos científicos.

Y para autorizar su presentación y evaluación por el tribunal correspondiente, expide el presente certificado en Salamanca, a 11 de Junio de 2014.

Dr. José Luis Revuelta Doval

Agradecimientos

Comenzar una carrera investigadora y preparar una tesis doctoral no es algo que se pueda agradecer en unas cuantas líneas ni a unas cuantas personas, pues es el fruto de una trayectoria vital impulsada por una serie de decisiones mas o menos afortunadas basadas unas veces en hechos, otras en personas y algunas otras en el puro azar de las circunstancias. No obstante, haciendo un resumen simplista y dejándome en el tintero gran cantidad de nombres dedico estas palabras a las personas que especialmente en estos cuatro años de tesis habéis estado ahí, a veces para enseñar, otras para aprender y algunas otras y de forma no menos importante "tan solo" habéis estado ahí.

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II. Introducción y Antecedentes

1. Biotecnología blanca e ingeniería metabólica de sistemas

La biotecnología es un término muy amplio que se refiere a toda aplicación tecnológica que utilice sistemas biológicos, organismos vivos o sus derivados para crear o modificar productos o procesos para usos específicos (Convention on Biological Diversity, Article 2. Use of Terms, United Nations. 1992).

Por tanto, se trata de una ciencia multidisciplinar que se ha subdividido en los llamados colores de la biotecnología según su ámbito de aplicación (DaSilva 2012). Estos tipos de biotecnología son los siguientes:

- Biotecnología roja: hace referencia a aquellas aplicaciones de la biotecnología relacionados con la biomedicina.
- Biotecnología verde: son aquellas aplicaciones relacionadas con el campo de la agricultura y las plantas.
- Biotecnología blanca: también se llama biotecnología industrial. Hace referencia a los procesos industriales que en general persiguen ser mas ecológicos y energéticamente favorables usando materias primas renovables y procesos de fermentación microbianos.
- Biotecnología azul: trata del uso de recursos marinos para generar productos de interés industrial.
- Biotecnología gris: engloba aquellos procesos biotecnológicos relacionados con el medio ambiente y la biorremediación.
- Otros colores menos extendidos de la biotecnología son: el amarillo (biotecnología alimentaria), marrón (utilización de los recursos de ecosistemas desérticos), dorado (relacionado con el sector bioinformático), negro (contra-bioterrorismo), púrpura (aspectos legales) y naranja (aspectos divulgativos).

Esta tesis doctoral se engloba en el ámbito de la biotecnología industrial o biotecnología blanca, ya que trata del uso de un microorganismo, *Ashbya gossypii,* y su modificación genética para convertirlo en un organismo capaz de llevar a cabo procesos de interés industrial.

Esta rama de la ciencia está ganando importancia en los últimos años, impulsada principalmente por la necesidad de utilizar productos renovables, en lugar de combustibles fósiles, y disminuir la acumulación de CO_2 atmosférico. El uso de derivados del petróleo produce efectos indeseables ya que:

1) Es muy contaminante y contribuye al calentamiento global.
2) Su fuente es limitada y se produce su agotamiento.
3) Su precio es inestable y la tendencia es alcista.
4) Se extrae de áreas del planeta políticamente inestables.
5) Genera graves desastres ecológicos cuando hay vertidos o fugas.

Así, los productos microbianos, que van desde compuestos químicos de interés (Chen and Nielsen 2013; Lee et al. 2012a) hasta biocombustibles (Alper and Stephanopoulos 2009; Peralta-Yahya et al. 2012; Zhang et al. 2011), son una alternativa prometedora al uso de recursos fósiles como el petróleo.

Dentro de la microbiología industrial podemos diferenciar dos grandes ramas del conocimiento, la ingeniería del microorganismo y la ingeniería del proceso industrial. Este trabajo se centra en la primera de las ramas, la ingeniería de microorganismos, y esto se lleva a cabo mediante un conjunto de técnicas que se engloban en la denominada ingeniería metabólica de sistemas. Éste término reciente hace referencia a la búsqueda de la optimización del metabolismo de un organismo para producir un compuesto de interés industrial (Lee et al. 2011; Lee et al. 2012a; Lee et al. 2012b; Sagt 2013). Es, por tanto, un campo multidisciplinar (ver figura 1) que incluye:

1) Técnicas de ingeniería genética y de proteínas,
2) Aplicaciones de biología sintética,
3) El uso de predicciones basadas en modelos matemáticos y
4) El análisis global aportado por las técnicas de biología de sistemas.

Se espera que la aplicación combinada de todas estas tecnologías emergentes permita un salto cuantitativo en las aplicaciones biotecnológicas de los microorganismos industriales, entre los que se encuentra el que es objeto de este trabajo: *Ashbya gossypii*.

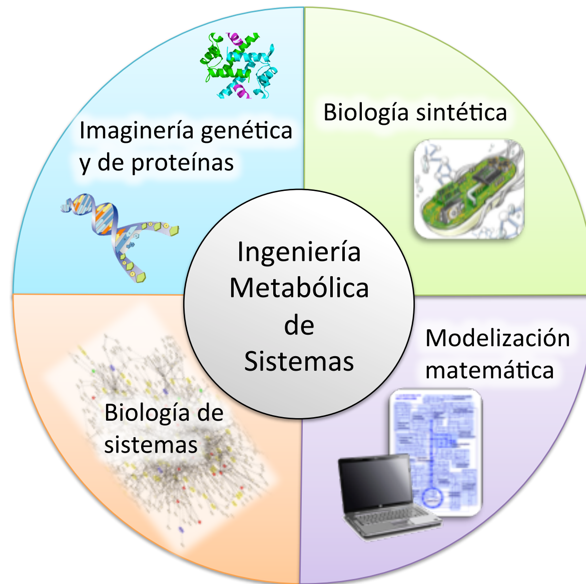

Figura 1: Definición gráfica de la ingeniería metabólica de sistemas. Se trata de una ciencia multidisciplinar integrada por la ingeniería genética y de proteínas, la biología sintética, la biología de sistemas y la modelización matemática del metabolismo.

2. *Ashbya gossypii*

Taxonomía

A. gossypii es un hongo filamentoso de la familia *Saccharomycetaceae*, perteneciente al grupo de los hemiascomicetos (Prillinger et al. 1997). Aunque pertenece al género *Eremothecium* y por tanto su nombre taxonómico actual es *Eremothecium gossypii*, por mantener una coherencia con la literatura publicada y en consenso con la comunidad científica que trabaja con este organismo, en el desarrollo de esta tesis se hablará de este hongo como *Ashbya gossypii*.

Breve historia

A. gossypii fue descrito originalmente en 1926 por Ashby and Nowel como el "hongo de la estigmatomicosis" (Ashby and Nowell 1926). Este organismo se identificó como el fitopatógeno causante de la estigmatomicosis en la planta de algodón *Gossypum hirsutum*. La infección de la planta se lleva a cabo a través de laceraciones causadas por insectos heterópteros, principalmente de los géneros *Antestia* y *Dysdercus* (Batra 1973). La

dependencia de estos vectores para la correcta infección permitió controlar fácilmente la enfermedad mediante la aplicación de insecticidas. Unos años después se identificó a este hongo como un productor natural de riboflavina, lo que impulsaría su estudio y aplicación en el campo de la biotecnología, como se detalla posteriormente.

Ciclo de vida

Actualmente existe controversia sobre el tipo de reproducción de *A. gossypii*. La hipótesis mas aceptada indica que la cepa silvestre tiene tipo sexual *MAT*a, pero carece de parte de los genes implicados en la ruta de la feromona. Por tanto, las esporas originadas serían del tipo asexual mientras que la existencia de un posible ciclo sexual no ha sido aún demostrada (Wendland et al. 2011; Wendland and Walther 2005).

El ciclo de vida de este organismo (ver figura 2) comienza con un estadio de espora acueliforme que presenta un apéndice característico en la parte posterior y que tiene función adhesiva, facilitando su propagación. La espora es uninucleada y haploide. En condiciones ambientales favorables tiene lugar la germinación, donde se da un crecimiento isotrópico seguido de la emisión de la primera hifa germinal polinucleada, originando un desarrollo polarizado. Poco tiempo después tiene lugar la aparición de la segunda hifa, dando lugar a un patrón de crecimiento bipolar. El micelio se desarrolla primero por ramificaciones laterales (micelio juvenil) y después por ramificaciones dicotómicas (micelio maduro). El agotamiento de los nutrientes del medio provoca el cese del crecimiento micelial que es seguido de la autolisis de algunas hifas y la formación de sacos esporíferos en otras. En estos sacos esporíferos se desarrollan las esporas, que una vez maduras son liberadas al medio para reiniciar así el ciclo de vida de *A. gosspyii* (Wendland and Philippsen 2000; Wendland and Walther 2005).

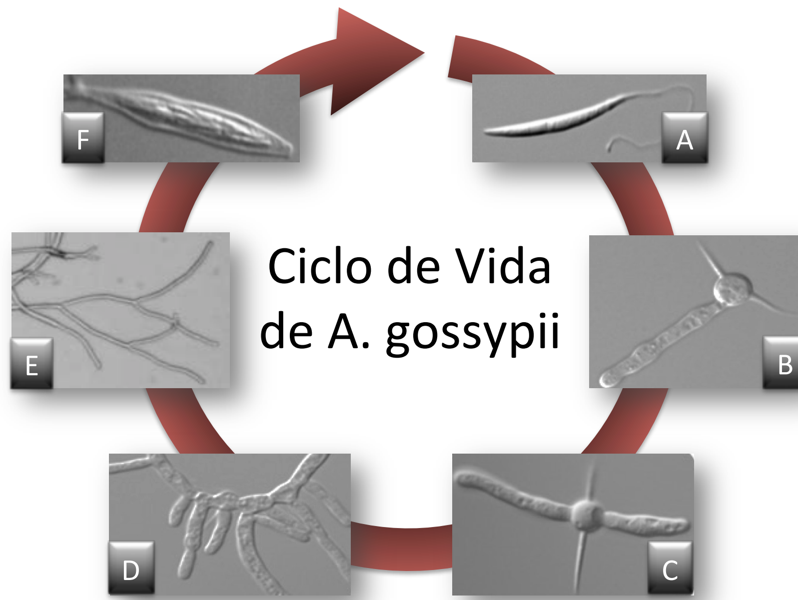

Figura 2: Ciclo de vida de *A. gossypii*. Los estadios que se muestran en la figura son: A) espora; B) germinación por crecimiento isotrópico y desarrollo de la primera hifa germinal (crecimiento polarizado); C) emisión de la segunda hifa (crecimiento bipolar); D) micelio juvenil formado por ramificaciones laterales; E) micelio maduro formado por ramificaciones dicotómicas y F) saco esporífero.

Organismo modelo en estudios de desarrollo filamentoso

A. gossypii ha sido utilizado como organismo modelo para investigar los factores implicados en el crecimiento de los hongos filamentosos. La sencillez de su uso en el laboratorio, junto con el reducido tamaño de su genoma y su cercanía evolutiva a la levadura unicelular *Saccharomyces cerevisiae*, han permitido identificar nuevas proteínas y establecer nuevos modelos relacionados con el desarrollo filamentoso. Se encuentra fuera del objetivo de esta tesis analizar los aspectos del desarrollo de este organismo, pero una revisión reciente permite profundizar en este tema (Wendland and Walther 2005).

3. *Ashbya gossypii*: Un microorganismo industrial

Años después de la identificación de *A. gossypii* como agente patógeno del algodón se descubrió su capacidad natural de producir grandes cantidades de riboflavina o vitamina B_2 (Wickerman et al. 1946). Como se detalla en apartados posteriores, en la actualidad este organismo es el mayor productor mundial de riboflavina (Kato and Park 2012). Esta capacidad natural del hongo ha permitido su explotación a nivel industrial, concluyéndose que su uso en fermentaciones a gran escala resulta económicamente muy ventajoso.

A. gossypii presenta ventajas a todos los niveles de un proceso industrial; antes de la fermentación o procesos "upstream", en la fermentación y después de la fermentación o procesos "downstream". En la figura 3 se muestra un diagrama de flujo que resume las etapas de un proceso fermentativo en *A. gossypii*.

Ventajas antes de la fermentación

Entre las ventajas del uso de este organismo en los procesos que tienen lugar antes de la fermentación destaca su capacidad de crecer en medios de cultivo económicos. Así, *A. gossypii* no solo es capaz de usar fuentes de carbono tradicionales como la glucosa sino también puede usar glicerol (Ribeiro et al. 2012), aceites (Stahmann et al. 1997) y productos de desecho de otros procesos industriales (Ming et al. 2003).

En la actualidad el glicerol es una fuente de carbono muy barata ya que es un subproducto de las plantas productoras de biodiesel, las cuales están cada vez mas extendidas (Almeida et al. 2012; da Silva et al. 2009). Su capacidad para utilizar aceites le permiten crecer en fuentes de desecho de otras plantas como sería, por ejemplo, el aceite de pino generado en las industrias papeleras que ya ha sido usado con anterioridad para otras biotransformaciones (Conner et al. 1976). Otro ejemplo es su capacidad de crecer en ABE ("activated bleaching earth") que es un material cerámico absorbente que se usa para eliminar carotenos, clorofilas y otros compuestos no deseados en el refinado de aceites vegetales. Estos ABE, que una vez usados son desechados, suponen solo en Japón 80000 toneladas métricas al año y son capaces de acumular hasta el 40% de su peso en aceites. Se ha comprobado en numerosos trabajos publicados que *A. gossypii* es capaz de crecer y producir grandes cantidades de riboflavina utilizando como única fuente de carbono estos baratos productos de desecho (Ming et al. 2003; Park and Ming 2004; Tajima et al. 2009).

Ventajas en la fermentación

A. gossypii, además, tiene la ventaja de haber sido utilizado en fermentaciones a gran escala, por lo que los parámetros más relevantes para su escalado son conocidos y, en cierta medida, extrapolables a otros procesos (Stahmann et al. 2000). Gracias a esto tanto los procesos de escalado como las condiciones óptimas de crecimiento están muy desarrollados. Este aspecto resulta especialmente importante si se tiene en cuenta que la gran mayoría de los procesos biotecnológicos que se intentan implantar con nuevos organismos fracasan en su proceso de escalado (Reisman 1993). Algunas veces el fallo es debido a que el microorganismo, que se comporta de la manera deseada a escala de laboratorio, no lo hace del mismo modo en cultivos mas grandes. Otras veces, la limitación viene impuesta por la tecnología actual que imposibilita el crecimiento del microorganismo en grandes volúmenes, como es el caso de los prometedores cultivos de microalgas (Armenta and Valentine 2013).

Ventajas tras la fermentación

No obstante, las mayores ventajas industriales de *A. gossypii* son aquellas que se encuentran en los procesos que tienen lugar después de la fermentación o ”downstream”. En primer lugar, al ser un organismo filamentoso, es muy rentable económicamente eliminar las células del producto deseado, ya que esto puede llevarse a cabo por simple filtración. Este proceso es inviable en bacterias y levaduras unicelulares y requieren de costosos procesos de centrifugación. Otra ventaja es que *A. gossypii* sufre autolisis al final de su crecimiento, y en estadios mas tempranos la autolisis se puede inducir a relativamente bajas temperaturas (40ºC)(Kurth 1990; Stahmann et al. 2000). Esto permite extraer de una forma sencilla compuestos que se producen en el interior celular, algo que a menudo en otros organismos es difícil ya que se requiere la ruptura mecánica, enzimática o química de las células, lo cual es costoso en los dos primeros casos y altamente contaminante en el último. Además, como ocurre en otros hongos filamentosos, *A. gossypii* puede llevar a cabo la llamada “fermentación en pellet”, que reduce la viscosidad del cultivo mejorando el mezclado y la transferencia de masa, lo que conlleva a una apreciable reducción de energía y costes (Zheng et al. 2012).

En conjunto, todas las características industriales descritas con anterioridad, convierten a *A. gossypii* en un organismo idóneo en biotecnología desde el punto de vista de la ingeniería del proceso.

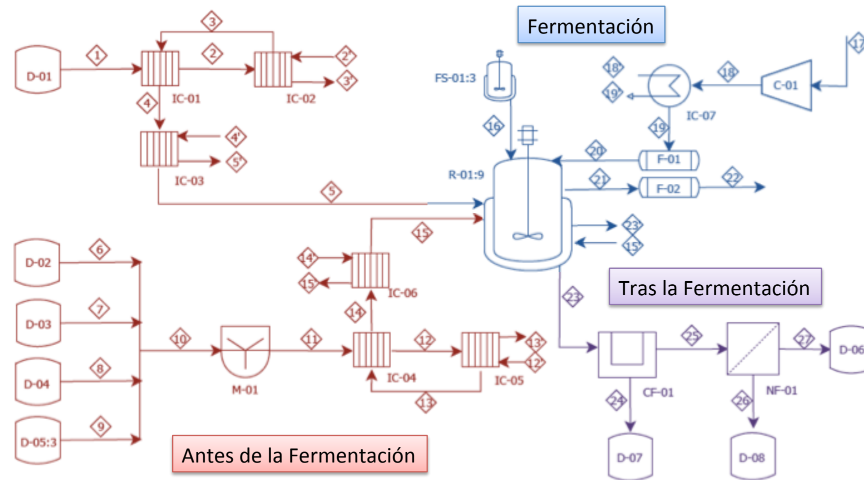

Figura 3: Diagrama de flujo de un proceso fermentativo de *A. gossypii*. En rojo se pueden ver los procesos que tienen lugar antes de la fermentación, que son los relativos a la preparación del medio de cultivo. Los nutrientes se almacenan en los depósitos (D-01:D-05), se mezclan en un mezclador (M-01) y se esterilizan en un sistema de tres intercambiadores de calor (IC-01:IC-06), que llevan la temperatura de la mezcla hasta la temperatura de esterilización y luego la devuelven a la temperatura de operación de reacción, en este caso 28ºC. En azul se pueden apreciar las etapas de la fermentación, donde aparecen los fermentadores de siembra (FS-01:3) que se inoculan en los bioreactores (R-01:9), las corrientes de entrada de aire y salida de gases y la refrigeración del reactor, necesaria para mantener una temperatura constante durante la fermentación. Los procesos que tienen lugar tras la fermentación se encuentran marcados en morado y comprenden todas aquellas etapas de separación y purificación del producto, en el caso tomado como ejemplo son una centrífuga (CF-01) y un equipo de nanofiltración (NF-01), necesarios para obtener nucleósidos.

4. *Ashbya gossypii*: Un organismo modelo para ingeniería metabólica y biotecnología

Además de las características industriales de *A. gossypii* arriba mencionadas, este hongo se puede considerar un organismo idóneo para realizar procesos de ingeniería metabólica.

La ingeniería metabólica es la manipulación mediante técnicas de ingeniería genética y de proteínas de una ruta metabólica y que generalmente tiene como objetivo la producción de un compuesto de interés o la eliminación de un agente contaminante. Para que en un organismo pueda realizarse ingeniería metabólica es importante que su genoma

sea conocido y se hayan desarrollado técnicas de biología molecular que permitan su modificación genética.

Un genoma ideal

El conocimiento de la secuencia genómica de un organismo es esencial para que éste pueda ser utilizado como modelo en aplicaciones de ingeniería metabólica. El genoma de *A. gossypii* fue secuenciado por primera vez en 2004 (Dietrich et al. 2004) y hoy día está totalmente disponible en la web: *agd.vital-it.ch/* (Gattiker et al. 2007; Hermida et al. 2005). Además, el genoma de *Ashbya gossypii*, que tiene 9,2 Mb y se organiza en 7 cromosomas, representa el genoma mas pequeño descrito hasta la fecha para un organismo eucariota (Dietrich et al. 2004). Esta característica es esencial para que en este organismo se puedan llevar a cabo estudios de esencialidad de reacciones enzimáticas que son necesarias para la mínima unidad de vida eucariota; lo que tendría importantes aplicaciones futuras como la creación de un organismo eucariota sintético. El genoma de *A. gossypii* guarda una sorprendente similitud con el del organismo modelo *S. cerevisiae*, de forma que hasta el 95% de los genes de *A. gossypii* tienen al menos un gen homólogo en la levadura (Dietrich et al. 2004). Además, mas del 90% de los genes comparten sintenia con la levadura, es decir que mantienen el orden de los genes y su orientación relativa (Dietrich et al. 2004). No obstante, *S. cerevisiae* presenta 5570 proteínas mientras que *A. gossypii* tan solo cuenta con 4718. Esto se debe a que *S. cerevisiae* pertenece al grupo de los hemiascomicetos que se originó después de un evento de duplicación general del genoma (Wolfe and Shields 1997). La innegable similitud entre ambos genomas ha servido para estudiar no solo el fenómeno de duplicación general del genoma sino también otros eventos evolutivos (Dietrich et al. 2013; Finlayson et al. 2011; Hall et al. 2005; Ling et al. 2014; Wendland and Walther 2011; Wendland and Walther 2014). Desde un punto de vista biotecnológico, la gran similitud con *S. cerevisiae*, el microorganismo eucariota mas estudiado, es una gran ventaja ya que permite extrapolar parte del conocimiento y herramientas generados para este organismo en *A. gossypii*.

Herramientas moleculares

Otro requisito para usar un organismo en ingeniería metabólica es que éste sea susceptible de ser modificado genéticamente; de tal manera que cuantas mas herramientas moleculares haya disponibles mas eficazmente se podrán desarrollar proyectos de ingeniería metabólica .

En este aspecto, en *A. gossypii* se han desarrollado desde hace un par de décadas distintas aproximaciones de ingeniería genética y biología molecular que permiten su modificación genética con el fin de: 1) Sustituir promotores nativos por otros mas fuertes/débiles o inducibles/constitutivos (Jimenez et al. 2008b; Mateos et al. 2006) 2) Eliminar genes determinados sustituyendo el gen elegido por un marcador de resistencia a antibióticos (Mateos et al. 2006); 3) Integrar genes heterólogos de forma estable en el genoma de *A. gossypii* (Ledesma-Amaro et al. 2014); 4) Construir plásmidos episómicos (Wright and Philippsen 1991); 5) Utilizar marcadores de selección variados, desde auxotrofías a antibióticos (Jimenez et al. 2008a; Kaufmann 2009); 6) Implementar marcadores de selección reciclables basados en el sistema Cre-*Lox*P (Ledesma-Amaro et al. 2014) 7) Y marcar proteínas con etiquetas fluorescentes (Kaufmann 2009). Un ejemplo de estas técnicas puede verse resumido en la figura 4.

De forma general, el eficiente mecanismo de recombinación homóloga de este organismo permite utilizar de manera eficaz en *A. gossypii* casi cualquier tecnología molecular desarrollada en otros organismos.

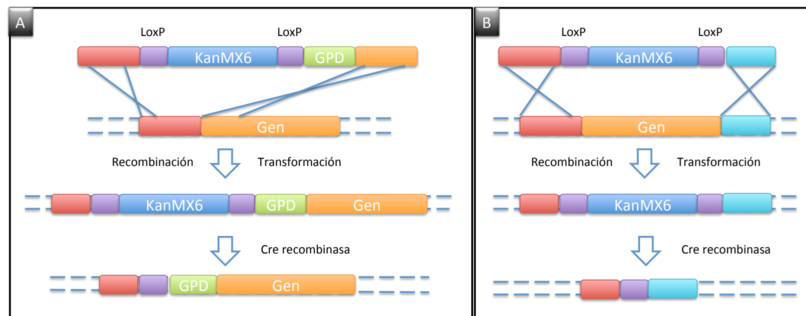

Figura 4: Ejemplo de técnicas de ingeniería genética en *A. gossypii*. A) Proceso de sobreexpresión de un gen por recombinación homóloga. Primero se integra la construcción de sobreexpresión por las regiones homólogas al genoma de *A. gossypii* previamente diseñadas, de tal manera que el gen original queda bajo el control del promotor fuerte *GPDp*. Después, mediante la expresión de la recombinasa Cre se elimina el marcador de selección *Kan*MX6 mediante recombinación de los sitios *Lox*P. B) Proceso molecular de deleción de un gen. En este caso la recombinación homóloga tiene lugar de tal manera que el marcador reemplaza la secuencia del gen. De igual forma que en la sobreexpresión, posteriormente se elimina el marcador mediante el sistema Cre-*Lox*P.

5. Aplicaciones industriales de *Ashbya gossypii*

Producción de riboflavina

Pocos años después del aislamiento de *A. gossypii* se describió su capacidad para producir riboflavina (Wickerman et al. 1946), vitamina responsable de su color amarillento (Figura 5).

Desde entonces este hongo ha sido optimizado por técnicas biotecnológicas hasta convertirse en el mayor productor mundial de riboflavina, sustituyendo a la síntesis química, mas costosa y contaminante. En la actualidad, de los 8000 toneladas métricas anuales que se producen de esta vitamina por procesos fermentativos, 4000 provienen del uso de *A. gossypii* (Park et al. 2011). El resto son producidos de forma minoritaria por procesos de fermentación con *Candida famata* y *Bacillus subtilis*.

En nuestro laboratorio se han desarrollado numerosos trabajos mejorando cepas de *A. gossypii* para la producción industrial de riboflavina. Esto ha originado publicaciones científicas y patentes, estando varias de ellas bajo explotación por la multinacional BASF. La mayoría de estos trabajos se han centrado en potenciar mediante ingeniería metabólica la ruta de purinas impulsando la síntesis de GTP, el precursor limitante en la síntesis de esta vitamina. Por un lado, se han sobreexpresado y desregulado los primeros genes de la ruta de síntesis *de novo* que codifican enzimas con actividad PRPP (fosforibosil pirofosfato) amidotransferasa (AGL334W) y PRPP sintasa (AGR371C y AGL080C). Estas modificaciones provocan un aumento en la producción de riboflavina de 10 veces y 1,8 veces respectivamente (Jimenez et al. 2005; Jimenez et al. 2008a). También se consiguió un incremento en la producción de la vitamina por la sobreproducción de glicina intracelular, un aminoácido esencial en la ruta de purinas. Esto se llevó a cabo mediante la sobreexpresión de la treonina aldolasa (*GLY1*) (Monschau et al. 1998) y la deleción de la serina hidroximetiltransferasa (*SHM2*) (Schlupen et al. 2003). Además, tanto la deleción completa del regulador de la ruta de purinas Bas2 como la eliminación del dominio BIRD de esta proteína, aumentó la producción de riboflavina 6 y 12 veces respectivamente (Mateos et al. 2006). También se han desarrollado estrategias de ingeniería metabólica para facilitar la secreción de la riboflavina mejorando su posterior extracción. Así, la deleción de la subunidad A de la ATPasa vacuolar Vma1, permitió la completa excreción de la vitamina al exterior celular, evitando la acumulación vacuolar de la misma (Forster et al.

1999). Recientemente, en nuestro laboratorio se han construido cepas con mayor capacidad de producción por la sobreexpresión de genes de la ruta de recuperación de purinas, ruta que permite generar directamente GTP a través de la incorporación de nucleótidos extracelulares.

Además de las cepas generadas por ingeniería racional, también se han obtenido cepas superproductoras de riboflavina mediante técnicas de mutagénesis aleatoria. De este modo, se han aislado cepas mejoradas mediante el uso de luz ultravioleta, mutagénesis química y el empleo *in vivo* de una variedad de la DNA polimerasa incapaz de reparar errores (Sanchez et al., sin publicar)

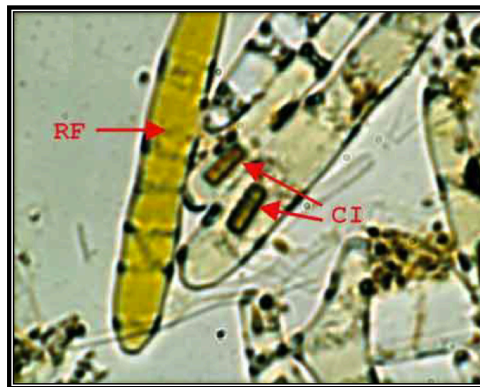

Figura 5: Células en cultivo de *A. gossypii*. La flecha RF representa riboflavina en el interior celular. La flecha CI indica cristales intracelulares de riboflavina almacenados en las vacuolas. Imagen modificada de Lim et al (Lim et al. 2003).

Producción de proteínas recombinantes

Aunque la producción de riboflavina es la única aplicación a gran escala que se está llevando a cabo en la actualidad con *A. gossypii*, las comentadas ventajas industriales de este hongo han propiciado la investigación de nuevas aplicaciones biotecnológicas.

Así se ha propuesto a *A. gossypii* como un organismo productor de proteínas recombinantes de interés. De forma pionera, nuestro laboratorio ha patentado el uso de este hongo para producir proteínas, y como prueba de concepto se expresaron celulasas heterólogas (WO2001023576A1). Con posterioridad se ha descrito la producción de otras dos celulasas de *Tricoderma reseii* (Ribeiro et al. 2010) y de una beta-galactosidasa de *Aspergillus niger* en *A. gossypii* (Magalhães et al. 2014). Además, se ha mejorado la capacidad natural de *A. gossypii* para secretar proteínas por métodos de mutagénesis tanto aleatorios como dirigidos (Ribeiro et al. 2013). En los últimos año se ha estudiado con

detalle la capacidad de N-glicosilación de proteínas de este microorganismo encontrándose interesantes diferencias con el mismo proceso en *S. cerevisiae* (Aguiar et al. 2013). De estos estudios se puede deducir que es ventajoso producir proteínas en este organismo ya que, a diferencia de las levaduras, no se produce un exceso de glicosilación de las proteínas aunque mantiene los procesos de glicosilación y fosforilación que no tienen lugar en sistemas bacterianos y que, sin embargo, son necesarios para la funcionalidad de las proteínas eucarióticas. Además, a diferencia de los costosos cultivos de células de mamífero, los de *A. gossypii* son mas asequibles económicamente. A pesar de todas estas ventajas, aún hacen falta muchos estudios sobre la expresión de proteínas recombinantes en *A. gossypii* para poder evaluar su viabilidad real y valorar si su capacidad productiva es superior a la de otros organismos filamentosos usados con este fin, como son *Aspergillus*, *Fusarium*, *Mucor*, *Trichoderma*, *Penicillum* y *Rhizopus*.

Producción de etanol

También se ha propuesto recientemente a *A. gossypii* como productor de etanol usando glicerol como fuente de carbono. Dado que el glicerol es un producto de desecho de la industria del biodiesel la posibilidad de convertirlo en otro biofuel, como el bioetanol, es interesante. La capacidad natural de producir etanol desde glicerol de *A. gossypii* es tan alta como la de cepas mejoradas genéticamente con este fin de *S. cerevisiae*, con un rendimiento en ambos casos de 13-14 g de etanol/g de glucosa (Ribeiro et al. 2012). No obstante, este rendimiento sigue siendo aún bajo si se compara con el proceso bacteriano y, por tanto, debe ser mejorado por técnicas de ingeniería metabólica para que pueda establecerse un sistema de producción económicamente viable.

Producción de ácido fólico

Recientemente, trabajos realizados en nuestro laboratorio han generado cepas de *A. gossypii* que producen cantidades notables de ácido fólico. El ácido fólico, o vitamina B9, es un compuesto esencial para la salud humana que debe ser adquirido mediante la dieta. Hoy en día existe en numerosos países una normativa obligatoria de fortificación de alimentos con vitamina B9 para asegurar unos niveles saludables de esta vitamina en la población. El ácido fólico se sintetiza desde GTP, al igual que la riboflavina, siendo por tanto *A. gossypii* un prometedor organismo para su producción biotecnológica. Las cepas generadas acumulan hasta 300 veces mas ácido fólico que el tipo silvestre y poseen títulos de producción muy superiores a los descritos hasta la fecha en otros organismos. Es

previsible que los resultados obtenidos puedan ser mejorados en un futuro no solo con estrategias de ingeniería metabólica sino también a través de la optimización de las condiciones de cultivo y fermentación.

A lo largo de esta tesis doctoral se han aplicado técnicas de ingeniería metabólica de sistemas para mejorar tanto procesos productivos ya consolidados, como la síntesis de riboflavina, como para desarrollar nuevas aplicaciones biotecnológicas del hongo industrial *A. gossypii*, como son la producción de potenciadores del sabor y moléculas derivadas de ácidos grasos.

III. Hipótesis y Objetivos

1. Hipótesis

A. gossypii es un microorganismo idóneo para la biotecnología industrial no solo por sus características apropiadas en las fermentaciones a gran escala, como se ha discutido en el capítulo I.3, sino también por ser susceptible de realizar estrategias de ingeniería metabólica de sistemas, como se ha descrito en el capítulo I.4. A pesar de esto, en la actualidad el único proceso industrial que se está llevando a cabo usando este hongo es la producción de riboflavina. Por lo tanto, en el desarrollo de esta tesis doctoral se ha buscado no solo mejorar la capacidad productiva de riboflavina sino también ampliar el abanico de aplicaciones industriales que este hongo puede realizar. Para esto último, nos basamos en dos características importantes del hongo:

1) El metabolismo lipídico de este organismo es muy activo, de forma que tanto su fuente natural de carbono como su medio de cultivo en la fermentación industrial está constituido por aceites vegetales; lo que le convierte en un buen candidato para producir ácidos grasos de interés.

2) El metabolismo purinogénico de este organismo es también muy activo, permitiéndole producir grandes cantidades de riboflavina a partir del sustrato GTP. Esta capacidad purinogénica podría proporcionar las bases para canalizar el flujo metabólico a otros intermediarios de esta ruta con alto valor industrial, como son los potenciadores del sabor inosina y guanosina.

2. Objetivos

1. **Desarrollar técnicas de ingeniería metabólica de sistemas en *A. gossypii*.**

   A. gossypii ya tiene puesto a punto técnicas de ingeniería genética y de proteínas pero para llevar a cabo ingeniería metabólica de sistemas se requiere un modelo metabólico a escala genómica, técnicas de biología sintética y la integración de técnicas de biología de sistemas. Por lo tanto, este objetivo busca la reconstrucción y validación de un modelo matemático que describa el metabolismo del hongo, la integración de datos disponibles de transcriptómica en el modelo matemático y el desarrollo de técnicas de biología sintética que permita expresar múltiples genes en una sola transformación o simular complejos enzimáticos in vivo para mejorar el flujo metabólico en una ruta mediante la canalización de los sustratos enzimáticos.

2. **Generar cepas superproductoras de riboflavina mediante ingeniería metabólica de sistemas.**

   Con este objetivo se pretende mejorar los niveles de producción de riboflavina mediante el estudio combinado de 1) los genes RIB, que aunque han sido ya identificados, todavía no han sido objeto de estrategias sistemáticas de ingeniería metabólica, y 2) del aumento de la disponibilidad del precursor GTP mediante la canalización del flujo en la ruta de purinas.

3. **Crear cepas modificadas capaces de acumular grandes cantidades de ácidos grasos de interés industrial.**

   Este objetivo trata de profundizar en el conocimiento del metabolismo lipídico en A. gossypii, en modificarlo para convertirlo en organismo oleaginoso y en dirigir el flujo metabólico para acumular compuestos lipídicos de interés, como son los ácidos grasos poliinsaturados y el biodiesel.

4. **Construir cepas de *A. gossypii* por ingeniería metabólica capaces de producir inosina y guanosina.**

   La inosina y la guanosina son precursores industriales de potenciadores del sabor y, además, en los últimos años se les han atribuido propiedades inmunomoduladoras beneficiosas para la salud humana. El objetivo es modificar la ruta de purinas para canalizar el flujo de intermediarios metabólicos hacia estos nucleósidos de interés.

VI. Artículos/Articles

Listado de artículos/List of articles

Artículo 1:
Ledesma-Amaro R, Jimenez A, Santos M, Revuelta J. 2013. Microbial production of vitamins. Book chapter in: Microbial production of food ingredients, enzymes and nutraceuticals, Sawston, Cambridge, UK: Woodhead Publishing Series in Food Science. Technology and Nutrition No. 246.

Artículo 2:
Ledesma-Amaro R, Kerkhoven EJ, Revuelta JL, Nielsen J. 2014. Genome scale metabolic modeling of the riboflavin overproducer Ashbya gossypii. Biotechnology and bioengineering 111(6):1191-9.

Artículo 3:
Ledesma-Amaro R, Serrano-Amatriain C, Jimenez A, Revuelta JL. 2014. Engineering of riboflaving overproduction in A. gossypii. (*Enviado*)

Artículo 4:
Ledesma-Amaro R, Santos MA, Jimenez A, Revuelta JL. 2014. Strain design of Ashbya gossypii for single-cell oil production. Applied and Environmental Microbiology 80(4):1237-44.

Patente: Revuelta JL, Jimenez A, Ledesma-Amaro R. Fatty acid production in Eremothecium. European Patent: EP-Patentanmeldung Nr. 13 190 151.4; BASF-Az.: PF75879 (INV0075879).

Artículo 5:
Ledesma-Amaro R, Santos MA, Jimenez A, Revuelta JL. 2014. Tuning single-cell oil production in Ashbya gossypii by engineering the elongation and desaturation systems. Biotechnology and bioengineering. (*Aceptado: en prensa*)

Artículo 6:
Ledesma-Amaro R, Lozano-Martinez P, Revuelta JL. 2014. Synthetic biology and metabolic engineering to produce polyunsaturated fatty acids in the industrial fungus Ashbya gossypii. (*Manuscrito en preparación*)

Artículo 7:
Ledesma-Amaro R, Jimenez A, Santos MA, Revuelta JL. 2013. Biotechnological production of feed nucleotides by microbial strain improvement. Process Biochemistry 48:1263-1270.

Artículo 8:
Ledesma-Amaro R, Buey RM, Revuelta JL. 2014. Increased production of inosine and guanosine through metabolic engineering of the purine pathway in *Ashbya gossypii*. (*Enviado*)

Artículo 9:
Buey RM, Ledesma-Amaro R, Balsera M, Pereda JM, Revuelta JL. 2014. Increased riboflavin production by manipulation of inosine 5´-monophosphate dehydrogenase in *Ashbya gossypii.* (*Enviado*)

Artículo 1:

Ledesma-Amaro R, Jiménez A, Santos M, Revuelta J. 2013. Microbial production of vitamins. Book chapter in: Microbial production of food ingredients, enzymes and nutraceuticals, Sawston, Cambridge, UK: Woodhead Publishing Series in Food Science. Technology and Nutrition No. 246.

21

Microbial production of vitamins

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DOI: 10.1533/9780857093547.2.571

Abstract: This chapter is a short review of the production of vitamins with the main focus on the microbial fermentation processes that are currently being employed in the production of vitamins. The state of the art of the industry of vitamins is extensively analysed with a description of both chemical and biotechnological systems that have been developed for the production of each vitamin. Finally, future trends in the microbial production of vitamins are analysed and the recent advances in strain design for whole-cell production of vitamins are discussed.

Key words: biotechnology, metabolic engineering, microbial production, vitamin, vitamin production.

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21.1 Introduction

Vitamins are defined as organic compounds that are essential for normal growth and nutrition and are required in small quantities in the diet because they cannot be synthesized by the body. According to their chemical nature, they can be divided into two groups: water-soluble vitamins and fat-soluble vitamins. These vitamins are naturally synthesized in microorganisms and plants and are essential for the metabolism of all living organisms.

The absence of sufficient amounts of these compounds in the diet leads to different health problems, which is especially important in cultures in which a varied diet is lacking, and this lack affects not only humans but also farm animals, with a huge economic impact. Thus, over the past decades, a vast industry relating to the production of vitamins has been successfully developed around the world and today vitamins are produced industrially and used widely not only as food and feed additives, but also as cosmetics, therapeutic agents and health and technical aids.

Traditionally, such vitamins have been produced by organic chemical synthesis, but this often requires a high number of reactions, using expensive devices as well as solvents that are usually undesirable pollutants harmful

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to the environment. To overcome these drawbacks, biological production of vitamins has been developed, by identifying natural producer organisms, finding the most profitable culture conditions, scaling up production and optimizing downstream processes to extract the pure product. Nevertheless, the intrinsic limitations of most natural producers make some vitamin titers unable to compete with chemical synthesis. Thus, biotechnology emerges as an environmentally friendly way to increase vitamin production, enriching natural sources or creating new ones that are more suitable for industrial purposes. In this sense, 'green' biotechnology can produce crops with high vitamin contents, which can be extracted or used directly as a food source of vitamins, while 'white' biotechnology is able to modify microorganisms through genetic and metabolic engineering, turning them into vitamin producers (see Part I of this book).

Microbial production has several advantages since microbes grow fast, they do not have to rely on climatic conditions and seasons, they can be scaled up readily and they are not in competition with human food needs. Additionally, many disciplines and techniques are being developed by the scientific community to attempt to encourage the rapid improvement of 'white' biotechnology, such as metabolic engineering, bioinformatics, fluxomics, metabolomics, systems biology, synthetic biology, and so on. Most vitamins are still produced chemically, but the number of microbiologically produced products is increasing with time and, currently, lab-scale approaches to the modification of microorganisms with a view to producing large amounts of all kinds of vitamins are under way.

Throughout this chapter, we shall explore each vitamin within a group, depending on its chemical nature: water-soluble or fat-soluble. Brief basic notions about the vitamin molecule, its biological functions and dietary intake are discussed and large-scale production is addressed, followed by some notes from some lab-scale studies that have provided promising results in terms of the microbial production of vitamins. Some previous chapters and reviews addressing similar topics can be considered for further reading.[1–6]

~~21.2 Fat-soluble vitamins~~

~~21.2.1 Vitamin and pro-vitamin A~~

~~Vitamin A is a group formed by different retinoids, retinol, retinal, retinoic acid and retinyl esters. Pro-vitamin A is composed of various carotenoids, the most important one being beta-carotene, while others are alpha-carotene and beta-cryptoxanthin. In our bodies, these pro-vitamins can be converted to retinal and retinoic acid, which are the active forms of vitamin A (Fig. 21.1).~~

~~Vitamin A is involved in many functions such as in the immune system, vision, reproduction, cellular communication, cell growth and~~

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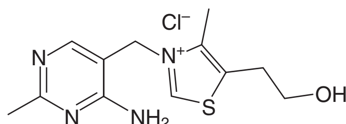

Fig. 21.5 Chemical structure of thiamine.

thiamine triphosphate (AThTP) and adenosine thiamine diphosphate (AThDP). The phosphorylated forms are thought to be the active forms of the vitamin, while thiamine (Fig. 21.5) is mainly the transportation form.

Thiamine has essential metabolic functions and deficiencies of this compound are associated with imbalances in carbohydrate status because it is involved in oxidative decarboxylation and transketolase reactions. It is also an active molecule of the nervous system. It can be found in the diet, especially in wheat germ, soy beans, dried beans and peas. Although it is widespread in foodstuffs, its concentration is often low because it is destroyed when food is cooked. Therefore, in developed countries rice and flour are usually fortified with this vitamin.

Vitamin B1 is produced chemically to supply human and animal needs (4200 t/a in 1996).[2] Over time, two methods of synthesis have been developed: (1) condensation of the pyrimidine and thiazole rings and (2) construction of the thiazole ring on a preformed pyrimidine portion. Recently, a patent protecting the metabolic engineering of bacteria to accumulate high amounts of thiamine in media has been developed using *B. subtilis*.[28] The patent refers to species of Bacillaceae, Lactobacillaceae, Streptococcaceae, Corynebacteriaceae and Brevibacteriaceae, in which a microorganism containing a mutation that deregulates thiamine production and causes thiamine products to be released from the cell is described. Several strategies of overexpression and deregulation of the genes involved in precursor synthesis and pathway engineering, among others, have also been described.

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21.3.2 Vitamin B2

Vitamin B2 is also called riboflavin, which takes its name from its yellow colour (flavus). It is essential for the proper functioning of all the flavoproteins, since riboflavin is the central component of the FAD and FMN cofactors. These are involved in oxidation–reduction reactions, which are key activities in the energy metabolism of carbohydrates, fats, ketone bodies and proteins. Vitamin B2 is also involved in the metabolism of other vitamins such as B6, B3 and A, in glutathione recycling and homocysteine metabolism. The highest amounts of riboflavin in food can be found in crimini mushrooms and spinach, but also in asparagus, green beans, yogurt and cow's milk.

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Industrial riboflavin production is a paradigm of how biotechnology can turn a chemical synthesis into a bioprocess with significant cost reductions by employing a genetic and metabolic bioengineering approach. Chemically, it is produced from D-glucose by three different processes. More than 9000 t/a of riboflavin were produced in 2010, around 75% being used for feed additive and the rest for human food and pharmaceuticals (Hoffmann-La Roche, BASF, ADM, Takeda).[2] This compound is naturally produced by several microorganisms such as ascomycete fungi (*Ashbya gossypii, Eremothecium ashbyii*), by yeasts such as *Candida flaeri* and *Candida famata*, and also by bacteria such as *B. subtilis* and *Corynebacterium ammoniagenes*.

Several metabolic approaches have been developed in *B. subtilis* by overexpression of the gene cluster involved in riboflavin synthesis[29] and including multiple copies of these genes.[30] Other approaches have attempted to express heterologous genes involved in riboflavin accumulation but only modest results have been achieved[31] and some modifications guided by transcriptional analysis have afforded a strain able to accumulate 15 g l^{-1} riboflavin.[32] However, most metabolic bioengineering strategies have been carried out in the main industrial producer *A. gossypii*.[33] All six genes of the riboflavin synthetic pathway have been overexpressed and its use to improve riboflavin production patented (Fig. 21.6).[34] Some other genes

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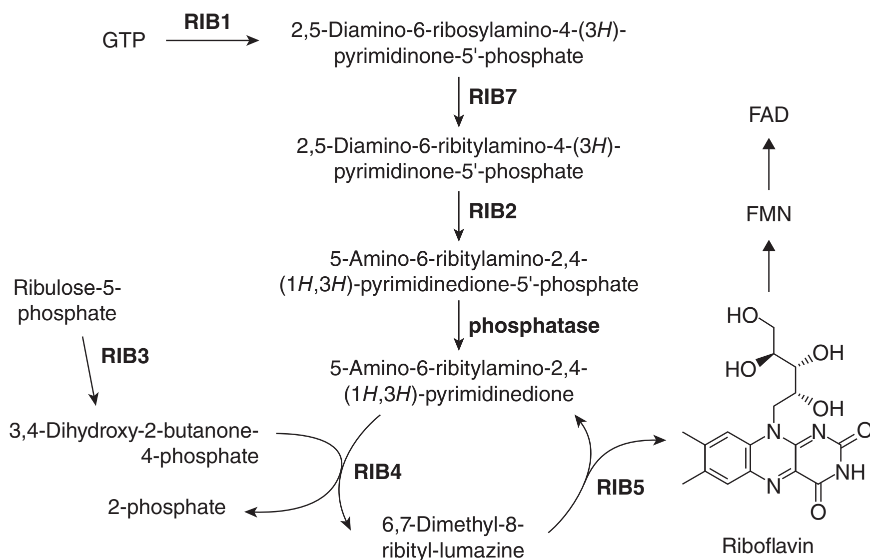

Fig. 21.6 Biosynthetic pathway of riboflavin in *Ashbya gossypii*. GTP = guanosine 5'-triphosphate; FAD = flavin adenine dinucleotide; FMN = flavin mononucleotide; RIB (1–5 and 7) = riboflavin biosynthesis gene(s).

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have been reported to accumulate the vitamin when they are overexpressed, deregulated, or disrupted.[33] These genetic alterations lead to an accumulation ranging from 1.4- to ten-fold increases relative to the wild-type, affording strains able to produce more than 13 g l^{-1}. In recent years, efforts in metabolic bioengineering have also been performed using *C. famata* and strains accumulating 4.1-fold the wild-type amount of the vitamin have been constructed.[35]

21.3.3 Vitamin B3

Vitamin B3 is a group formed of nicotinic acid, nicotinamide and other compounds such as inositol hexanicotinate, that exhibit a related biological activity. These compounds can be clustered together under the term niacin (which sometimes is only used referring to nicotinic acid) (see chemical structure in Fig. 21.7).

Like all B vitamins, niacin is involved in energy metabolism, in the use of carbohydrates, fats and proteins, and it is therefore needed for healthy skin, hair, eyes, liver and nervous system. Niacin also helps the body's stress- and sex-related hormones. It is used, or is currently under investigation for use, in therapies against high cholesterol, atherosclerosis and heart disease, diabetes and osteoarthritis. This vitamin can be found in different foods but can also be produced from the essential amino acid tryptophan, which can be obtained from most sources of protein. To cite just some examples, niacin is present in liver, chicken and beef, seeds, mushrooms and yeast and also in some species of fish.

Nicotinamide and nicotinic acid are produced both chemically and biotechnologically (22,000 t/a, BASF, Lonza and Degussa).[2] The major use of this production is for animal nutrition and the remaining 25% is used for food enrichment and pharmaceutical applications. Chemically it is synthesized by the oxidation of 5-ethyl-2-methylpyridine or by total hydrolysis of 3-cyanopyridine. However, this latter transformation can also take place in a bioprocess using nitrilase (to produce nicotinic acid) or nitrile hydratase (to produce nicotinamide). Nitrilase has been overexpressed in *Rhodococcus rhodochrous*, generating a strain able to convert almost all 3-cyanopyridine into nicotinic acid.[36] Nitrile hydratase in *R. rhodochrous* exceeded 50% of the total cellular protein, permitting high production of nicotinamide from 3-cyanopyridine. Both reactions are almost stoichiometric, even

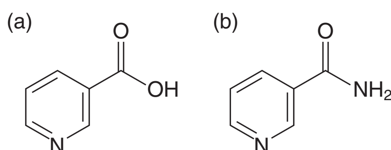

Fig. 21.7 Chemical structure of nicotinic acid (a) and nicotinamide (b).

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~~developed in order to analyse the huge amounts of data that these system sciences provide us with.~~

~~Currently there are some vitamins that are almost exclusively produced by microorganisms, such as vitamin B2 and B12. Vitamins C and A are produced both chemically and microbiologically and certain others such as Vitamin D, K, B3 and B5 have at least one or more microbial enzymatic step. The rest, which are mostly produced chemically, have been studied in depth in order to develop biological platforms to make their microbial production competitive enough to replace their chemical synthesis. This has been described previously, vitamin by vitamin, in the most recent publications and patents in microbial biotechnology. Thus these future developments in biotechnology should lead us to more sustainable, environmentally-friendly and economically competitive vitamin production systems using microorganisms as effective cell factories.~~

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Article

Biotechnology and Bioengineering

Genome Scale Metabolic Modeling of the Riboflavin Overproducer *Ashbya gossypii*

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ABSTRACT: *Ashbya gossypii* is a filamentous fungus that naturally overproduces riboflavin, or vitamin B2. Advances in genetic and metabolic engineering of *A. gossypii* have permitted the switch from industrial chemical synthesis to the current biotechnological production of this vitamin. Additionally, *A. gossypii* is a model organism with one of the smallest eukaryote genomes being phylogenetically close to *Saccharomyces cerevisiae*. It has therefore been used to study evolutionary aspects of bakers' yeast. We here reconstructed the first genome scale metabolic model of *A. gossypii*, iRL766. The model was validated by biomass growth, riboflavin production and substrate utilization predictions. Gene essentiality analysis of the *A. gossypii* model in comparison with the *S. cerevisiae* model demonstrated how the whole-genome duplication event that separates the two species has led to an even spread of paralogs among all metabolic pathways. Additionally, iRL766 was used to integrate transcriptomics data from two different growth stages of *A. gossypii*, comparing exponential growth to riboflavin production stages. Both reporter metabolite analysis and in silico identification of transcriptionally regulated enzymes demonstrated the important involvement of beta-oxidation and the glyoxylate cycle in riboflavin production.

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KEYWORDS: *Ashbya gossypii*; riboflavin; genome-scale metabolic model; *Eremothecium*; systems metabolic engineering; microbial biotechnology

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Introduction

Ashbya gossypii (or *Eremothecium gossypii*) is a filamentous hemiascomycete with unique features which make it a widely studied organism in both basic research (Wendland and Walther, 2005) and industrial applications (Kato and Park, 2012).

Its genome, which has been sequenced and annotated (Dietrich et al., 2004; Hermida et al., 2005), is one of the smallest eukaryotic genomes with only 4,718 protein-coding genes. It is highly similar to the well-studied organism *Saccharomyces cerevisiae* and 95% of *A. gossypii* genes have homologs in yeast (Dietrich et al., 2004; Fig. 1A). In spite of this high homology, the *S. cerevisiae* genome encodes over 800 additional genes (5,570 in total), many of which have presumably originated from a whole-genome duplication event (Wolfe and Shields, 1997). While most components are shared between the two genomes, the life cycles of these two organisms show important differences, such as the probable lack of a sexual cycle and the multinucleated hyphae development in *A. gossypii*. It has therefore been considered a model organism to study the minimal eukaryotic free-living cell (Finlayson et al., 2011), the event of genome duplication (Wolfe and Shields, 1997), the pheromone signal cascade and sexual cycle (Wendland et al., 2011), fungal development and polarized hyphal growth (Schmitz and Philippsen, 2011) and to reinvestigate the *S. cerevisiae* genome (Brachat et al., 2003).

A. gossypii is a natural overproducer of riboflavin (vitamin B2), which animals and humans cannot synthesize. Riboflavin is a high value industrial compound used not only in pharmaceuticals, but also in animal feed additives, cosmetics and in the food industry (Shi et al., 2009). *A. gossypii* has been considered a paradigm of the industry-friendly white biotechnology and it is nowadays one of the world's top producers of riboflavin by microbial fermentation (Kato and Park, 2012; Ledesma-Amaro et al., 2013b; Stahmann et al., 2000). The availability of the genome sequence and genetic engineering tools (Wendland et al., 2000) have allowed the development of metabolic engineering approaches that have significantly increased the vitamin production titer (Jimenez

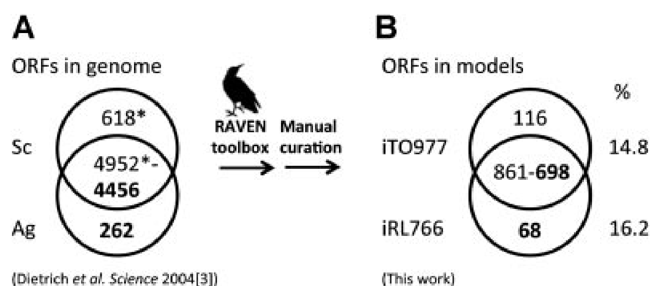

Figure 1. Comparison between the *A. gossypii* and *S. cerevisiae* genomes and the iTO977 and iRL766 models. (**A**) Comparison of the total ORFs in the genomes of *S. cerevisiae* (Sc) and *A. gossypii* (Ag). (**B**) Comparison of the genes in the yeast model iTO977 and the *Ashbya* model iRL766, after extensive manual curation. The overlapping regions of (A) and (B) represent the number of homologous genes. The numbers in bold are *A. gossypii* genes with homologs in *S. cerevisiae* while normal numbers are genes from *S. cerevisiae* with homologs in *A. gossypii*. Asteriks denote approximate value.

et al., 2005, 2008; Park et al., 2011). In addition to its well-established use in riboflavin production, the capabilities of *A. gossypii* to produce proteins (Ribeiro et al., 2010, 2013) and bioethanol from glycerol (Ribeiro et al., 2012) are being investigated.

Genome-scale metabolic models (GEMs) have become an important tool in the understanding of metabolic networks and they have been useful in both the study and the applications of biological systems (Garcia-Albornoz and Nielsen, 2013; Kim et al., 2012; Ledesma-Amaro et al., 2013a). GEMs can serve to describe novel metabolic networks, find essential genes and metabolites, predict substrate consumption capabilities and aid in re-annotating genomic information (Costanzo et al., 2010; Ibarra et al., 2002; Vongsangnak et al., 2008). Additionally, they can be used as a scaffold to integrate omics experiments and thus the physiological differences between different conditions can be analyzed in the context of the metabolic network (Osterlund et al., 2013). Thirdly, GEMs have been widely used in the biotechnology field for identification of metabolic bottlenecks and novel metabolic engineering targets (Agren et al., 2013b; Caspeta and Nielsen, 2013; Otero et al., 2013; Park et al., 2007b).

Here we present the first curated genome-scale metabolic model of the filamentous fungus *A. gossypii*, called iRL766. Along this work iRL766 was validated and its capabilities to predict biomass growth, riboflavin production and substrate utilization were shown. Gene essentiality was studied in the context of its own network and of its close relative *S. cerevisiae*, enlightening the event of the whole genome duplication. Due to the industrial interest in this organism for riboflavin production, the biosynthetic pathway of the vitamin within the model was studied in more detail. Finally the metabolic network was used to integrate transcriptomics data and transcriptionally controlled reactions in the transition from a trophic phase to a riboflavin productive phase, representing possible targets for further strain engineering.

Materials and Methods

Reconstruction of Genome-Scale Metabolic Model

The first step in reconstructing the iRL766 model was to create two draft models using the RAVEN toolbox (Agren et al., 2013a). The first draft model was based on protein orthology between the *S. cerevisiae* model iTO977 (Osterlund et al., 2013) and *A. gossypii*. For this, homology between the protein coding sequences of the *S. cerevisiae* and *A. gossypii* genomes were evaluated using bi-directional BLASTp, with the following cut-offs: E-value $<1e-30$, identity $>40\%$ and alignment length >200 amino acids. All 1-1 orthologs were included, to capture potential paralogs while ensuring that only genes that map back to the original gene in the BLASTp in the opposite direction are included. For the second draft model the *A. gossypii* genome was queried using hidden Markov models (HMMs), which were constructed from Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2012) consensus sequences, as described in (Agren et al., 2013a). The protein sequence of *A*. gossypii strain ATCC10895 (http://genolevures.org/) was used for construction of both draft models. The two draft models were merged to generate a model representing reactions with and without homology in *S. cerevisiae*, using the naming convention and charge balancing from iTO977. Subsequently, reactions in the combined model were manually curated to assure correctness with respect to reaction stoichiometry, metabolites and co-factors involved, and to identify gaps in the metabolic network. Manual curation was performed using KEGG, MetaCyc (Caspi et al., 2012), Saccharomyces Genome Database (Cherry et al., 2012), Ashbya Genome Database (Gattiker et al., 2007), and available literature. Where *A. gossypii* specific knowledge of gene-associations, metabolic reactions and localization was insufficient, data from the closely related *S. cerevisiae* was used.

The biomass equation and ATP for maintenance were taken from the iTO977 model, as insufficient information is available from *A. gossypii*. When oil was used as a carbon source, the biomass equation was modified in lipid composition according to previously reported data (Stahmann et al., 1994).

All simulations were performed using RAVEN and COBRA toolboxes in Matlab. iRL766 is available in the BioMet Toolbox (Cvijovic et al., 2010; http://sysbio.se/BioMet/—will be uploaded upon acceptance of paper).

Constraints-Based Flux Analysis and Simulations

Flux balance analysis was widely performed used in simulations with the reconstructed GEMs (Park et al., 2009). Loops were removed from the solution by minimizing the number of reactions carrying flux. To simulate the trophic phase, either glucose or oleic acid uptake rates were set, while biomass production was set as objective function. To simulate the riboflavin production phase, the consumption rate of lipid bodies was set, while riboflavin production was set as

objective function. ATP for maintenance was not considered in the productive phase since we assumed that this is produced from different molecular sources (as mutants unable to consume lipid bodies have the same growth pattern as wild type (Ledesma-Amaro R., personal communication). Experimental data were extracted from previous publications using EasyNData (http://puwer.web.cern.ch/puwer/EasyNData/).

Gene Essentiality Predictions

In silico evaluation of single gene knock-outs were performed using the RAVEN toolbox, which takes the presence of isoenzymes into account. Lethality was estimated according to a relative fitness value (f = mutant growth rate/wild type growth rate), when f was smaller than the cutoff value (0.9) the gene was considered essential (Osterlund et al., 2013). Less strict cutoff values did not drastically reduce the number of lethal genes (Supplementary File 7). Two different glucose-based media were used, minimal media (MM) and rich media (YPD). Minimal media consisted of the uptake of glucose, ammonium, oxygen, sulfate and phosphate while YPD media also allowed the uptake of amino acids and nucleotides.

In Silico Prediction of Carbon and Nitrogen Utilization

Assessment of growth on different carbon sources was performed by constraining the uptake of each carbon source individually to 6 C-mol/gCDW/h and maximizing for cell growth. In silico growth rates were qualitatively compared to experimental growth rates, and therefore categorized as: no growth, reduced growth (0.001–0.090 h^{-1}) and normal growth (0.090 h^{-1}). Experimental data were classified in the same three categories.

Integrative Data Analysis

Normalized microarray dataset E-MEXP-1945 (Gattiker et al., 2007) was obtained from ArrayExpress (www.ebi.ac.uk/arrayexpress/). Eighteen and 103 h samples were analyzed, corresponding to fast growing advanced mycelia and sporulating mycelia, respectively. The sporulating mycelia represent the riboflavin productive stage, as sporulation is a process directly correlated to vitamin production (Stahmann et al., 2001). These data were integrated in the model iRL766 in order to identify reporter metabolites and transcriptionally regulated key enzymes. Reporter metabolites were identified with R package Piano (Varemo et al., 2013). A random sampling algorithm was applied in order to identify transcriptionally regulated enzymes (Bordel et al., 2010). Normalized array data as described above were used as input. The upper and lower bounds of the exchange fluxes and biomass or riboflavin reactions in iRL766 were constrained according to experimentally measured fluxes for each condition (Supplementary file 5). A set of 500 flux distributions was generated for each of the considered conditions (Osterlund et al., 2013). Z-scores were calculated from the two sets of flux distributions and transformed into probabilities of change by using the cumulative Gaussian distribution, as described in Bordel et al. (2010). The values of the moderated t-statistic from the transcriptomics data were used to calculate a probabilistic score P for each reaction representing the probability that the flux and the transcription are significantly changed in the same direction between the two conditions (cutoff $P > 0.9$).

Results and Discussion

Reconstruction and Comparative Analysis of the *A. gossypii* Metabolic Network

The high sequence similarity between *S. cerevisiae* and *A. gossypii* allowed us to create a first draft model using the yeast model iTO977 as template (Osterlund et al., 2013), using bi-directional BLASTp, containing 725 genes. No homologs could be found for 69 genes from iTO977, corresponding to 76 reactions (Supplementary file 3). Among these were reactions involved in biotin and myo-inositol synthesis, rendering *A. gossypii* auxotrophic for both nutrients (Demain, 1972). Additionally, 12 reactions related to galactose metabolism were absent in *A. gossypii*, explaining the incapability of this organism for growth using galactose as a sole carbon source (Kurtzman et al., 2011). The use of a *S. cerevisiae* model as a template has the disadvantage that the resulting draft model can only contain genes, and their associated reactions, that have high sequence identity in *A. gossypii*. To include the function of *A. gossypii* genes that were not represented in the *S. cerevisiae* genome, a second draft model was generated based on similarity with KEGG consensus sequences (Kanehisa et al., 2012), containing 570 genes. KEGG consensus sequences provided only 11 *A. gossypii* genes with no homologs in *S. cerevisiae*, responsible for 30 reactions. Eleven of these reactions were predicted to require riboflavin derivatives, such as flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD). These reactions were added to the first draft model, by transferring the reactions indicated in Supplementary file 3. Subsequent extensive manual curation of the merged model generated the model iRL766. iRL766 included 766 genes which were involved in 1,595 reactions, comprising 799 unique metabolites distributed in four different compartments (cytoplasm, mitochondria, peroxisome, and extracellular). After manual curation, the number of unique genes in each model increased to 116 and 69 for the iTO977 and iRL766 models, respectively. Although the model has less annotated genes than iTO977 (977 genes) they still constitute 16.2% of the total open reading frames of the genome, which is a higher coverage than for the yeast model (14.8%; Fig. 1B).

While some differences between the models were observed, it is unlikely that these are sufficient to explain

the unique properties of each strain but a combination of these and the unequal regulation at several biological levels.

Riboflavin Biosynthetic Pathway in iRL766

A. gossypii is used on a large-scale in the bioindustry for riboflavin production, often using glucose (Jimenez et al., 2008) or oils (Park et al., 2011) as carbon source. The oils used are typically rich in triacylglycerols, predominantly composed of oleic acid (triolein). To investigate the production of riboflavin from glucose and triolein, FBA was performed using either carbon source and optimized for riboflavin production (Supplementary file 4 and Fig. 2).

When glucose was used as sole carbon source, 86 reactions were involved in riboflavin production (Fig. 2A, Supplementary file 4). The most important pathways activated in the model were glycolysis, TCA cycle and oxidative phosphorylation, to provide the energy supply, followed by purine metabolism and pentose phosphate pathway to provide the precursors GTP and ribulose-5-phosphate respectively, and finally riboflavin metabolism to synthesize the active vitamin.

When oleic acid was used as carbon source a total of 134 reactions were involved in riboflavin synthesis (Fig. 2B, Supplementary file 4). The activities of glycolysis and the pentose phosphate pathway were reduced as fatty acid degradation was now involved in substrate utilization. Precursors and riboflavin biosynthesis reactions remained almost invariant during growth on both carbon sources.

These pathways described by iRL766 are in concordance with previously published data (Park et al., 2007a, 2011; Stahmann et al., 2000; Sugimoto et al., 2009). Flux variability analysis was performed to search for alternative solutions (Supplementary file 10), and these results adhere to the same pathways as shown in Figure 2.

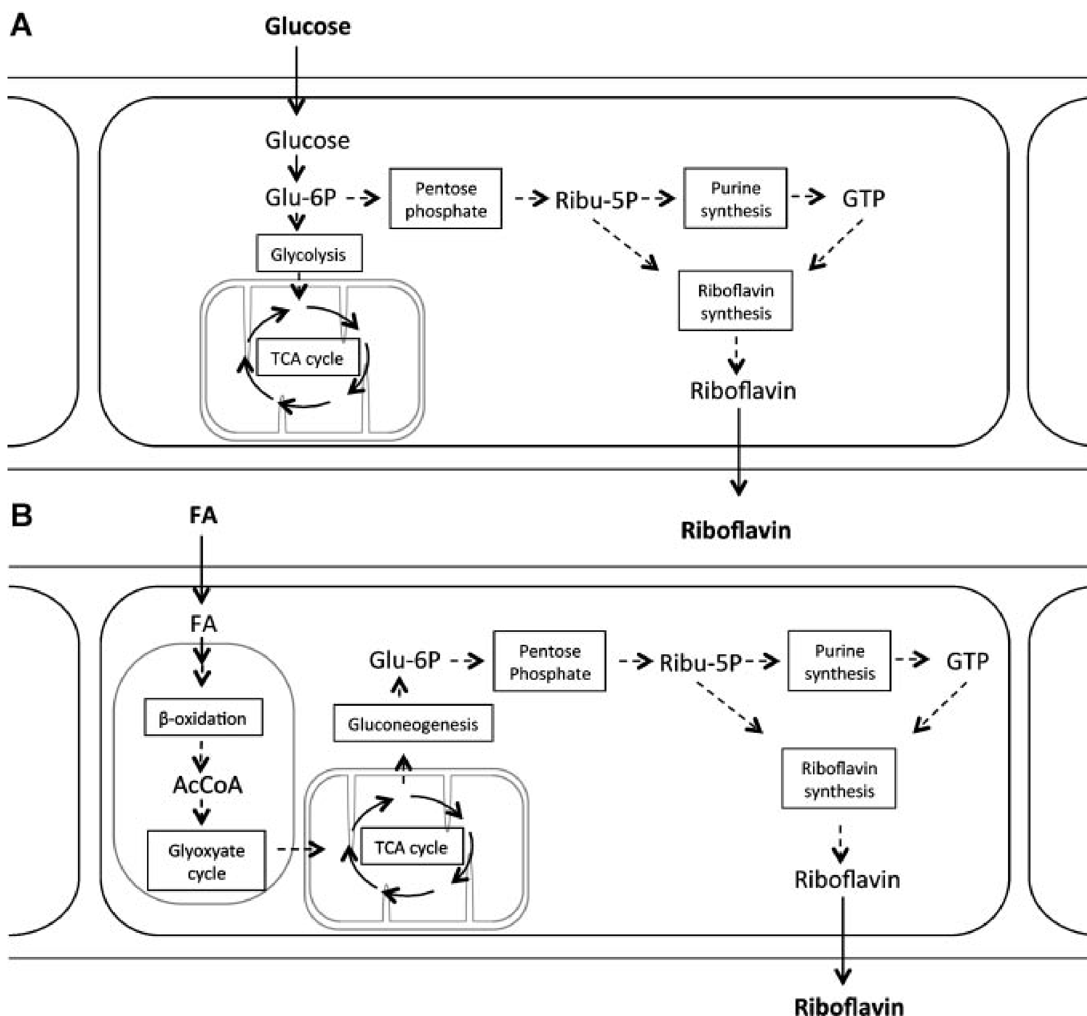

Figure 2. Scheme of pathways that carry flux when the riboflavin production phase is simulated in iRL766 when (**A**) glucose or (**B**) oleic acid is used as carbon source. FA, fatty acid; AcCoA, acetyl coA; TCA, tricarboxylic; Glu-6P, glucose-6-phosphate; Ribu-5P, ribulose-5-phosphate.

Growth Rate and Riboflavin Synthesis Predictions

A. gossypii has two growth stages, a first trophic phase, when cells grow exponentially while riboflavin production is minimal; and a second phase, or productive phase when cells stop growing and riboflavin is overproduced (Mateos et al., 2006). These two conditions were simulated in the model, to validate the growth rate and riboflavin production predictions with experimental data (Supplementary file 5). The growth predictions on both glucose and triolein were in good concordance with experimental data (Fig. 3).

There is limited comprehensive data available that can be used to validate the riboflavin productive phase. Firstly, the carbon source used for the vitamin production has not been entirely elucidated. In most experimental approaches, glucose and oils have been exhausted from the media at the end of the exponential phase. It has been hypothesized that fatty acids, accumulated as lipid bodies during growth, are remobilized in the productive phase permitting riboflavin synthesis, supported by microscopy and Nile red staining of *A. gossypii* lipid bodies (Lim et al., 2003; Stahmann et al., 1994, Ledesma-Amaro et al., 2013c). This hypothesis is further supported by an increase in riboflavin synthesis when oils are used for growth, as *A. gossypii* accumulates at least twice the amounts of lipids during the first growth phase in these conditions (Stahmann et al., 1994). Using experimental lipid bodies consumption rate to constrain the model and riboflavin production as objective function resulted in a vitamin production rate of 0.0156 mmol/gCDW/h, which was close to the experimental value of 0.0126 mmol/gCDW/h (Stahmann et al., 2001).

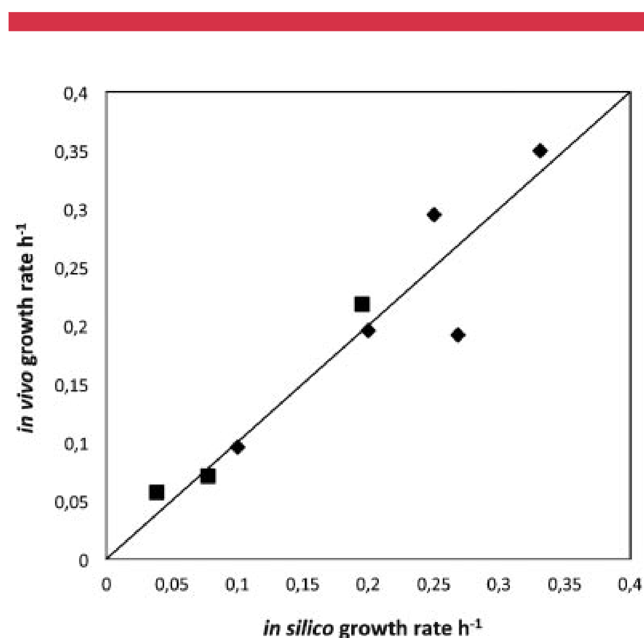

Figure 3. Comparison between the in silico prediction of specific growth rate with experimental data. Growth phenotypes were collected from literature and compared with simulated values when glucose (diamonds) and oils (squares) were used as carbon sources (Supplementary file 5). Black line represent perfect correlation.

In Silico Prediction of Carbon and Nitrogen Utilization

The model was further validated by comparison with experimental growth data on different carbon and nitrogen sources (Kurtzman et al., 2011; Ribeiro et al., 2012; Table I and Supplementary file 6). Of the 41 tested carbon and nitrogen sources, 39 showed comparable growth in both model and experiments, while only D-ribose and D-xylose were identified in the model as false positives. While *A. gossypii* is capable of growth with D-ribose as sole carbon source, it can do so only in concentrations below 0.3% due to possible toxicity, which cannot be predicted by the model (Revuelta, J.L. personal communication). Regarding D-xylose, it was recently found that while the machinery for D-xylose consumption is encoded in the *A. gossypii* genome, as demonstrated in iRL766, D-xylose can only be converted to xylitol and not metabolized further (Ribeiro et al., 2012). Even though the putative genes for xylose catabolism are present, they cannot be expressed to sufficient level to ensure growth, as it is the case in *S. cerevisiae* (Scalcinati et al., 2012; van Zyl et al., 1989).

In Silico Analysis of Reaction Essentiality

As *A. gossypii* has one of the smallest eukaryotic genomes (Dietrich et al., 2004), iRL766 represents an interesting platform to study gene essentiality and the minimal set of reactions to maintain a free-living eukaryotic cell. Additionally, *A. gossypii* shares high homology to the well-studied yeast *S. cerevisiae*, while baker's yeast encodes 852 more genes within its genome, due to the whole-genome duplication event that took place in baker's yeast but not in *A. gossypii* (Dietrich et al., 2004). We compared gene essentiality in iRL766 to iTO977 to investigate this WGD event.

In both models, iTO977 and iRL766, each gene was knocked out sequentially, and the effect on the specific growth rate was examined in minimal and rich media (MM and YPD). As anticipated, the fraction of essential genes in the model increased from 18.9% in yeast to 22.5% in *A. gossypii* in MM, and from 12.4% in yeast to 14.2% in *A. gossypii* in YPD (Fig. 4A). Many of the differences between the two species can be explained by reactions that are associated with a single gene in iRL766, whereas they are associated with two or three different genes in iTO977 (e.g., IMD3-IMD4). Essential genes for the two models were grouped according to their metabolic function (Fig. 4B, Supplementary file 7). Many essential genes were found in lipid metabolism and oxidative phosphorylation, followed by sugar and nucleotide metabolism. In MM also many genes in amino acid metabolism were found to be essential, while the presence of amino acids in YPD allowed auxotrophs to scavenge amino acids from the growth medium. There were no major differences in the spread of essential genes among the different metabolic pathways in the two models, indicating that paralogs in *S. cerevisiae* are evenly spread out over all metabolic functions.

Table I. Carbon and nitrogen utilization.

	Predicted value	Experimental value	Refs.
Carbon source			
Acetate	o	o	Mickelson (1950)
Cellobiose	oo	o	Kurtzman et al. (2011)
Cellulose	—	—	Farries and Bell (1930); Ribeiro et al. (2012)
Citrate	o	o	Kurtzman et al. (2011)
D arabinose	—	—	Kurtzman et al. (2011)
D glucosamine	—	—	Kurtzman et al. (2011)
D mannitol	—	—	Kurtzman et al. (2011)
D ribose	oo	—	Kurtzman et al. (2011)
D xylose	oo	—	Kurtzman et al. (2011); Ribeiro et al. (2012)
D sorbitol	oo	o	Kurtzman et al. (2011)
D gluconate	o	o	Kurtzman et al. (2011)
DL lactate	—	—	Kurtzman et al. (2011)
Erythritol	—	—	Kurtzman et al. (2011)
Ethanol	oo	o	Kurtzman et al. (2011); Mickelson (1950)
Fructose	oo	oo	Farries and Bell (1930)
Galactitiol	—	—	Kurtzman et al. (2011)
Galactose	—	—	Kurtzman et al. (2011)
Glucose	oo	oo	Kurtzman et al. (2011); Ribeiro et al. (2012)
Glycerol	oo	oo	Kurtzman et al. (2011); Ribeiro et al. (2012)
Hexadecane	—	—	Kurtzman et al. (2011)
Inulin	—	—	Kurtzman et al. (2011)
L arabinose	—	—	Kurtzman et al. (2011); Ribeiro et al. (2012)
L rhamnose	—	—	Kurtzman et al. (2011)
L sorbose	—	—	Kurtzman et al. (2011)
Lactose	—	—	Farries and Bell (1930); Kurtzman et al. (2011); Mickelson (1950)
Maltose	oo	o	Kurtzman et al. (2011)
Melezitose	—	—	Kurtzman et al. (2011)
Melibiose	—	—	Kurtzman et al. (2011)
Methanol	—	—	Kurtzman et al. (2011)
Methyl-alpha-D glucoside	—	—	Kurtzman et al. (2011)
Myoinositol	—	—	Kurtzman et al. (2011)
NADglucosamine	—	—	Kurtzman et al. (2011)
Pyruvate	o	o	Mickelson (1950)
Raffinose	o	oo	Kurtzman et al. (2011)
Ribitol	—	—	Kurtzman et al. (2011)
Starch	oo	oo	Ribeiro et al. (2012)
Succinate	o	o	Kurtzman et al. (2011)
Sucrose	oo	oo	Kurtzman et al. (2011)
Trehalose	oo	oo	Kurtzman et al. (2011)
Nitrogen source			
NH4	oo	oo	Ribeiro et al. (2012)
Nitrate	—	—	Kurtzman et al. (2011); Ribeiro et al. (2012)

Comparison of in silico (predicted) and in vivo (experimenal) growth on different carbon and nitrogen sources. — represents no growth; o represents reduced growth; while oo represents unimpaired growth, compared to growth on glucose (Supplementary file 6).

Integrative Data Analysis

Another powerful use of GEMs is as a scaffold for the analysis of data from different biological levels. Focusing on riboflavin production, we analyzed previously published microarray data (Gattiker et al., 2007) from trophic stage and riboflavin productive stage Even though these microarray data have extensively been investigated for cell development (Rischatsch, 2007), the information they carry about riboflavin production has remained unexplored.

Reporter Metabolites

Patterns in the transcriptional responses of the metabolic network can be revealed by using the network topology from a GEM (Patil and Nielsen, 2005). We therefore used iRL766 to group genes into sets all coding for enzymes that catalyze reactions where a specific metabolite takes part. Enriched or significant gene sets (i.e., reporter metabolites) can be interpreted as metabolic hotspots or metabolites around which important transcriptional changes occur (Supplementary file 8). Among the most significant reporter metabolites were primarily amino acids, likely a consequence of the protein turnover that is observed between the two growth stages (Rischatsch, 2007). While riboflavin was among these top metabolites, its precursor cytosolic glycine appeared as the most reliable reporter metabolite. Glycine has been described as a key factor in riboflavin production and the increase of this, either by metabolic engineering approaches or media supplementation leads to riboflavin

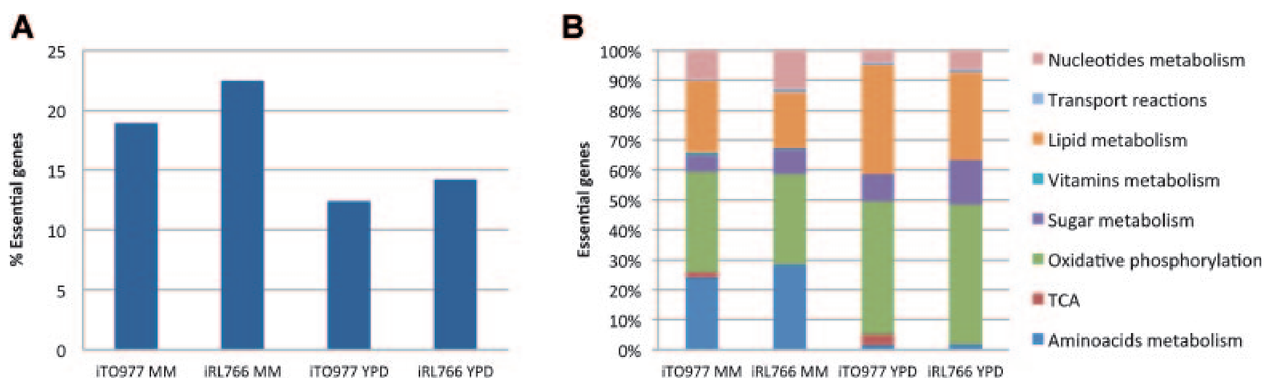

Figure 4. Gene essentiality was investigated in both models iRL766 and iTO977 by knocking out individual genes and assessing their growth rate. Whole models comparison where (**A**) the fraction of essential genes over all genes in the model, and (**B**) metabolic groups of these essential genes are shown. MM, minimal media: YPD, rich media.

overproduction (Monschau et al., 1998; Schlupen et al., 2003). Glyoxylate and the vast presence of fatty acids derivatives as reporter metabolites supported the proposed mechanism for riboflavin production through beta-oxidation of lipid bodies and the glyoxylate cycle. Also other intermediates in riboflavin biosynthesis appeared at high positions in the up regulated reporter metabolite list such as 4-(1-D-ribitylamino)-5-amino-2,6-dihydroxypyrimidine, 6,7-dimethyl-8-(1-D-ribityl)lumazine or 3,4-dihydroxy-2-butanone 4-phosphate.

Identification of Transcriptionally Regulated Enzymes

Genetic engineering of *A. gossypii* has allowed to increase the production of riboflavin to an industrial level (Kato and Park, 2012). Enzymes that correlate in expression levels and metabolic fluxes are potential targets for overexpression, in an attempt to direct the metabolic flux towards a desired product. We identified what changes in metabolic fluxes in response to the riboflavin production phase are transcriptionally regulated. The transcriptomics dataset is described above.

For the metabolic fluxes, a space of feasible flux distributions was defined by constraining the model by a small set of experimental metabolic fluxes and appropriate objective functions for each condition. A collection of possible flux distributions was calculated using a random sampling algorithm (Bordel et al., 2010). Flux distributions from the two conditions were compared and probability scores were calculated to identify the significance of the observed changes. The probability score of the change in metabolic flux was compared to the probability score of a transcriptional change, obtained from the microarray dataset. The reactions with a probability score higher than 0.9, as calculated by the random sampling algorithm (Bordel et al., 2010), present both changes in the same direction. Statistics for both up- and down-regulated reactions were calculated (Supplementary file 9).

In total, 38 reactions demonstrated correlated increases in both transcript levels and metabolic fluxes; these reactions were transcriptionally up-regulated. Four of those reactions were part of the riboflavin synthetic pathway: RIB2, RIB3, RIB4, and RIB5 (Fig. 5). Other up-regulated reactions were involved in beta-oxidation (e.g., POX and FOX2) and gluconeogenesis (FBP1, PCK1), supporting the hypothesis of lipid bodies consumption in the riboflavin productive phase. Almost all other up-regulated reactions were involved in extracellular uptake of amino acids and nucleosides. *A. gossypii* can suffer autolysis in the latest phases of its life cycle: some cells can lyse in order to supply nutrients to the remaining cells which continue their riboflavin overproduction and sporulation (Stahmann et al., 2000). This explains the up-regulation of amino acid and nucleoside permeases in this growth phase. All the genes involved in the reactions identified as up-regulated are potential candidates for overexpression in order to increase riboflavin production. While some, such as the RIB genes, are more obvious, especially the genes of beta-oxidation and gluconeogenesis

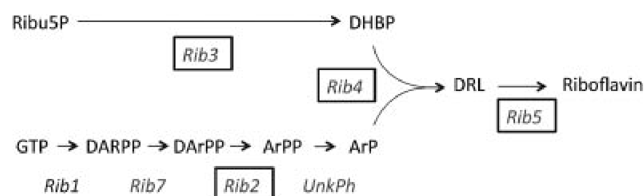

Figure 5. Transcriptionally controlled reactions (reactions where the change in flux correlate with change in expression of the involved gene) in riboflavin synthesis. Gene in squares are responsible for an up-regulated reaction identified by the random sampling algorithm when comparing trophic phase and riboflavin productive phase. UnkPh (unknown phosphatase), Ribu5P (ribulose 5 phosphate), DHBP (3,4-dihydroxy-2-butanone 4-phosphate), DARPP (2,5-diamino-6-hydroxy-4-(5′-phosphoribosylamino)-pyrimidine), DArPP (2,5-diamino-6-ribitylamino-4(3H)-pyrimidinone 5′-phosphate), ArPP (5-amino-6-(5′-phosphoribitylamino)uracil), ArP (4-(1-D-ribitylamino)-5-amino-2,6-dihydroxypyrimidine), DRL (6,7-dimethyl-8-(1-D-ribityl)lumazine).

would require further investigation to elucidate whether the sole overexpression of these genes lead to some increase in the vitamin titer and experimental data must be done to confirm this.

A larger number of reactions, 127, were transcriptionally down-regulated and many of those are related to biomass formation. Interestingly, many reactions from purine biosynthesis were down-regulated, despite this pathway's involvement in the supply of GTP for riboflavin production. This has been previously described for some of the genes of purine biosynthesis, such as ADE4 (Mateos et al., 2006), indicating that either the nucleotides have been synthetized in excess during the trophic phase or the nucleotides/nucleosides from partially broken mycelia are taken up from the environment using the up-regulated permeases. Additionally, down-regulation also prevents riboflavin consumption by riboflavin kinase (FMN1) and glycine degradation by SHM2 and GCV. Indeed the disruption of SHM2 in *A. gossypii* leads to an accumulation of riboflavin (Schlupen et al., 2003) and both SHM2 and ADE4 have been identified as down-regulated by the transcription factor BAS1 (Mateos et al., 2006).

Conclusions

In this study, we reconstructed and validated the first manually curated genome-scale model of *A. gossypii*, iRL766, with particular focus on the riboflavin production by this fungus. The model is in good concordance with existing experimental data on the pathways involved in riboflavin production, growth rate and the use of different carbon sources. Subsequently, the curated GEM was used to analyze gene essentiality, particularly in comparison to the *S. cerevisiae* GEM iTO977. The increased number of predicted essential genes in iRL766 could be transcribed to the whole genome duplication event that has occurred in *S. cerevisiae* but not in *A. gossypii*. Categorizing the predicted essential genes in metabolic pathways indicated that this event has led to an even spread of paralogs among all metabolic pathways. This is in agreement with the proposed rationale of gene duplication, where duplicated genes do not belong to any particular dominated function and do not occur more frequently in essential genes, but often overlap functional roles (Kuepfer et al., 2005).

Subsequently, the model was used as a scaffold to analyze microarray data to investigate the transcriptional changes related to riboflavin production. Reporter metabolite analysis indicated glycine, glyoxylate, and fatty acids as important metabolic hot spots, together with the anticipated riboflavin. A random sampling algorithm was employed to identify transcriptionally regulated reactions in the GEM. Together with the anticipated RIB genes, also beta oxidation and gluconeogenesis were transcriptionally up-regulated. Additionally observed important reporter metabolites and transcriptionally regulated reactions were supported by previous publications. This underwrites the validity of the model and indicates how it can be used in future approaches not only for overproduction of riboflavin, but also other vitamins, proteins, or lipids, as well as it can be used to improve our knowledge of this organism.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Artículo 4:

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Strain Design of *Ashbya gossypii* for Single-Cell Oil Production

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Single-cell oil (SCO) represents a sustainable alternative for the oil industry. Accordingly, the identification of microorganisms with either higher lipidogenic ability or novel capacities for the transformation of raw materials constitutes a major challenge for the field of oil biotechnology. With this in mind, here, we were prompted to address the lipidogenic profile of the filamentous hemiascomycete *Ashbya gossypii*, which is currently used for the microbial production of vitamins. We found that *A. gossypii* mostly accumulates unsaturated fatty acids (FAs), with more than 50% of the total FA content corresponding to oleic acid. In addition, we engineered *A. gossypii* strains both lacking the beta-oxidation pathway and also providing ATP-citrate lyase (ACL) activity to block the degradation of FA and to increase the cytosolic acetyl-coenzyme A (CoA) content, respectively. The lipidogenic profile of the newly developed strains demonstrates that the mere elimination of the beta-oxidation pathway in *A. gossypii* triggers a significant increase in lipid accumulation that can reach 70% of cell dry weight. The use of *A. gossypii* as a novel and robust tool for the production of added-value oils is further discussed.

The oil industry sustains the increasing demand for most carbon-based compounds, such as fuels, lubricants, functional polymers, and other high-value fine chemicals. In addition, some feed supplements, colorants, and nutraceuticals, such as carotenoids and polyunsaturated fatty acids (PUFAs), are also derived from lipids (1). Crude oil, as well as plant oil and animal fat, is the main source of oil for industry. However, fossil oil supplies are limited, and their use involves negative environmental consequences. Moreover, the use of oilseeds and animal fat for nonfood applications results in competition with food, higher prices, and other economic and environmental concerns (2–4).

In this context, microbial oil—also referred to as single-cell oil (SCO)—represents a sustainable alternative feedstock for the oleochemical industry. SCO accumulated by oleaginous microorganisms may have several advantages over other oil resources: the fermentative processes are independent of climate and, more importantly, the use of waste products as substrates avoids competition with edible resources and makes the process environmentally friendly (4, 5). In addition, the application of "white biotechnology" (i.e., industrial biotechnology) to fermentation processes allows strain design for the production of many different high-value oleochemicals (2, 4, 6).

An oleaginous microorganism is defined by its ability to accumulate more than 20 to 25% of the cell dry weight as lipid content. Additionally, it has been reported than some oleaginous yeasts are able to reach a level of lipid accumulation of up to 70 to 80% of their cell dry weight in oil-containing media (4, 7, 8).

The concept of lipid accumulation denotes a metabolic imbalance between lipid biosynthesis and degradation. Fatty-acid (FA) biosynthesis starts with the carboxylation of acetyl-coenzyme A (CoA) by acetyl-CoA carboxylase (ACC) to form malonyl-CoA. Next, the FA synthase (FAS) multienzymatic complex catalyzes the elongation of the acyl-CoA chain by condensing malonyl-CoA molecules and acetyl-CoA (8). Therefore, acetyl-CoA is the essential donor molecule for FA biosynthesis, and it has been reported that a continuous supply of cytosolic acetyl-CoA is crucial for lipid accumulation. Indeed, the presence of the enzyme ATP-citrate lyase (ACL), which catalyzes the formation of acetyl-CoA from citrate in the cytosol, is considered a common hallmark of all oleaginous microorganisms (8, 9). Unlike FA biosynthesis, beta-oxidation is the principal catabolic pathway for the degradation of FA and hence represents a competing pathway for lipid accumulation (10, 11). Beta-oxidation comprises a four-step oxidative cycle that removes two carbons (an acetyl-CoA molecule) from the acyl-CoA chain in each cycle. The pathway is present in all eukaryotes, although there is significant diversity in terms of substrate specificity, enzyme architecture, and subcellular localization among the different organisms (11).

Several strategies have been developed to enhance the accumulation of lipids in oleaginous microorganisms (10, 12, 13). Metabolic flux channeling toward lipid accumulation has mainly been succeeded by two different strategies: (i) increasing the cytosolic pool of acetyl-CoA by the overexpression of genes encoding FA synthesis-promoting activities, such as the *ACL* genes, and (ii) blocking the principal FA-degrading activities by deleting the genes controlling FA beta-oxidation (10, 12–15).

Ashbya gossypii is a filamentous hemiascomycete that belongs to the family Saccharomycetaceae and that naturally overproduces riboflavin (vitamin B_2). *A. gossypii* has been considered a paradigm of sustainable white biotechnology through its use in the industrial overproduction of riboflavin and other vitamins (16). Indeed, we have previously described metabolically engineered strains of *A. gossypii* that are able to produce 10-fold more riboflavin than a wild-type (WT) strain (17–19), and similar results have been achieved for the production of folic acid (J. L. Revuelta, unpublished results). The fermentation processes using *A. gossypii* have several advantages, such as the mycelial autolysis that occurs in late stationary phase, which avoids costly recovery

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steps, and its ability to grow in different oil sources and oil-containing wastes (16, 20).

According to general criteria, *A. gossypii* is considered to be a nonoleaginous microorganism (9). However, it has been reported that *A. gossypii* is able to accumulate between 10 and 20% of the cell dry weight as lipid bodies and, moreover, 54% of the total FA composition corresponded to the essential unsaturated omega-6 linoleic acid when soybean oil was used as the sole carbon source (21). In contrast to most bona fide oleaginous microorganisms, *A. gossypii* lacks any ACL-coding gene, and hence, cytosolic acetyl-CoA is provided solely by acetyl-CoA synthase (ACS) activity. Furthermore, *A. gossypii* has only one FA beta-oxidation pathway with a unique acyl-CoA oxidase gene (*AER358C-POX1*) that controls the first beta-oxidation reaction. Conversely, most oleaginous microorganisms, such as *Yarrowia lipolytica* or *Mucor circinelloides*, show two or three beta-oxidation pathways in several different cellular compartments and up to six different acyl-CoA oxidase-encoding genes (9, 15).

In the present study, we wished to investigate the potential ability of *A. gossypii* to accumulate significant amounts of SCOs in a biotechnologically feasible process. We show that the metabolic engineering of *A. gossypii* allows the isolation of oleaginous strains that are able to accumulate up to 70% of their biomass as lipid content. Additionally, the role of *ACL* genes as an essential feature of oleaginous microorganisms is examined. In sum, we report a novel microbial tool for the production of higher-added-value lipids by the rational design of metabolic engineering manipulations.

MATERIALS AND METHODS

***A. gossypii* strains, media, and growth conditions.** The *A. gossypii* ATCC 10895 strain was used and was considered a wild-type strain. The other *A. gossypii* strains used in the study are listed in Table S1 in the supplemental material. *A. gossypii* was cultured at 28°C using MA2 rich medium, synthetic complete (SC) medium, or synthetic minimal medium lacking leucine (SC−leu) (17). For lipid accumulation analyses, the carbon source of the MA2 medium was either 8% glucose or 1% glucose plus 2% oleic acid (Sigma), previously emulsified by sonication in the presence of 0.02% Tween 40 (22). Cultures with oil were centrifuged at 10,000 × *g* for 10 min, and the resulting cell pellet was washed three times with equal volumes of SB solution (9 g/liter NaCl in 0.5% bovine serum albumin [BSA]) (23). *A. gossypii* transformation, sporulation conditions, and spore isolation have been described elsewhere (24). Briefly, DNA was introduced into *A. gossypii* germlings by electroporation, and primary heterokaryon transformants were isolated in selective medium. Homokaryon transformant clones were obtained by sporulation of the primary transformants and isolation on antibiotic-containing plates. Genomic DNA and RNA isolation was carried out as previously described (17). Concentrations of 250 mg/liter for Geneticin (G418) (Gibco-BRL) and 100 mg/liter for nourseothricin (cloNAT; Werner Bioagents) were used where indicated. Liquid cultures were initiated either from mycelial overnight preinocula or with 1×10^6 spores per liter of medium and were performed on a rotary shaker at 200 rpm.

Lipid extraction and quantification by GC-MS. Fatty acid methyl esters (FAMEs) were extracted and transmethylated from freeze-dried *A. gossypii* biomass using a modification of the method described by Bligh and Dyer (25). One hundred micrograms of lyophilized mycelia was mixed with 1 ml of hexane (Sigma) and 750 μl of 2% sulfuric acid in methanol under a nitrogen atmosphere. The samples were incubated at 100°C for 90 min. Then, the samples were cooled on ice, and the transmethylation reaction was stopped by the addition of 0.5 ml of distilled water. The upper phase was recovered by centrifugation, and the extraction step was repeated by adding 0.5 ml of hexane to the lower phase. The two hexane-soluble phases were mixed and evaporated with nitrogen. The FAMEs were resuspended in 100 μl of hexane and used for gas chromatography-mass spectrometry (GC-MS) analysis.

GC-MS was carried out using an Agilent 7890A gas chromatograph with an Agilent MS200 mass spectrometer. A VF50 column (30 m long, 0.25-mm internal diameter, and 25-μm film) was used. The conditions for analysis were as follows: helium was used as the carrier at 1 ml/min, with a split ratio of 1:20. The oven program was as follows: an initial temperature of 90°C for 5 min, a ramp of 12°C/min up to 190°C, and a ramp of 4°C/min up to 290°C. Mass spectrometric detection was from 50 to 400 Da. FAs were identified by comparison with commercial fatty acid methyl ester standards (FAME32; Supelco) and quantified by an internal-standard method using 50 μg of $C_{17:0}$ (Sigma).

Gene deletion and gene overexpression. For gene deletions, a *loxP-KanMX-loxP* or a *loxP-NatMX-loxP* replacement cassette flanked by recombinogenic sequences for the corresponding target gene was used (data not shown). The *loxP* repeated inverted sequences enabled the selection marker to be eliminated and subsequently reused by expressing a Cre recombinase, as described elsewhere (26, 27). The selection markers with recombinogenic flanks for the deletion of *POX1* (*loxP-KanMX-loxP* module for G418 resistance) and *FOX2* (*loxP-NatMX-loxP* module for nourseothricin resistance) were obtained using the primers listed in Table S2 in the supplemental material. Genomic integration of the deletion modules was confirmed by analytical PCR and DNA sequencing (data not shown). For the heterologous overexpression of the *ACL* genes from *Y. lipolytica* (http://genolevures.org/yali.html#), each open reading frame (ORF) (*YALI0E34793g* and *YALI0D24431g*, here referred to as *ACL1* and *ACL2*, respectively) was PCR amplified from *Y. lipolytica* genomic DNA (the primer sequences are listed in Table S2 in the supplemental material) and verified by DNA sequencing. Each ORF was fused to the promoter and terminator sequences of the *A. gossypii GPD* (*AgGPD*) gene (for *ACL2*, the terminator of the *AgPGK1* gene was used) following a previously described cloning strategy (18, 28). The *ACL2* overexpression module comprising the recombinogenic flanks for the *AgSTE12* locus, the selection marker *loxP-KanMX-loxP*, the *ACL2* ORF, the *AgGPD* promoter, and the *AgPGK1* terminator sequences was assembled following a one-pot DNA-shuffling method using the sequence of the BsaI restriction enzyme in the acceptor vector, as previously described (28). For the *ACL1* overexpression module, the *ACL1* ORF was PCR amplified as an NdeI-BamHI fragment using the corresponding primers (see Table S2 in the supplemental material) and inserted between the *AgGPD* promoter and terminator sequences in an overexpression cassette containing the integration and selection modules, as previously described (18). The overexpression modules were isolated and purified by enzymatic restriction (SapI and PmeI for the *ACL2* and *ACL1* modules, respectively) and were used to transform spores of the corresponding *A. gossypii* strain. The *ACL2* cassette was integrated into the *STE12* locus, and the *ACL1* cassette was inserted into the *LEU2* locus of the genome (data not shown). For the construction of double mutants, the selection marker was eliminated in the single mutants by expressing a Cre recombinase, and the double mutant was obtained using the same selection marker (data not shown). The genomic integration of the overexpression modules was confirmed by analytical PCR and DNA sequencing (data not shown).

Acetyl-CoA quantification. *A. gossypii* mycelium (1 to 2 g) was lyophilized and pulverized mechanically. Then, samples (60 to 100 mg cell dry weight) were deproteinized by the addition of 6 μl of 1 N perchloric acid and sonicated on ice at medium intensity to avoid excessive heating. The homogenized extract was centrifuged at 13,000 × *g* to remove insoluble material and kept on ice. The supernatant (200 μl) was neutralized (pH 6 to 8) with three aliquots (20 μl each) of 3 M potassium bicarbonate solution, adding the aliquots while vortexing. The samples were cooled on ice and centrifuged to pellet the potassium bicarbonate. Twenty to 50 μl of the samples was used to quantify the acetyl-CoA content using an Acetyl-Coenzyme A Assay Kit (Sigma) following the manufacturer's instructions.

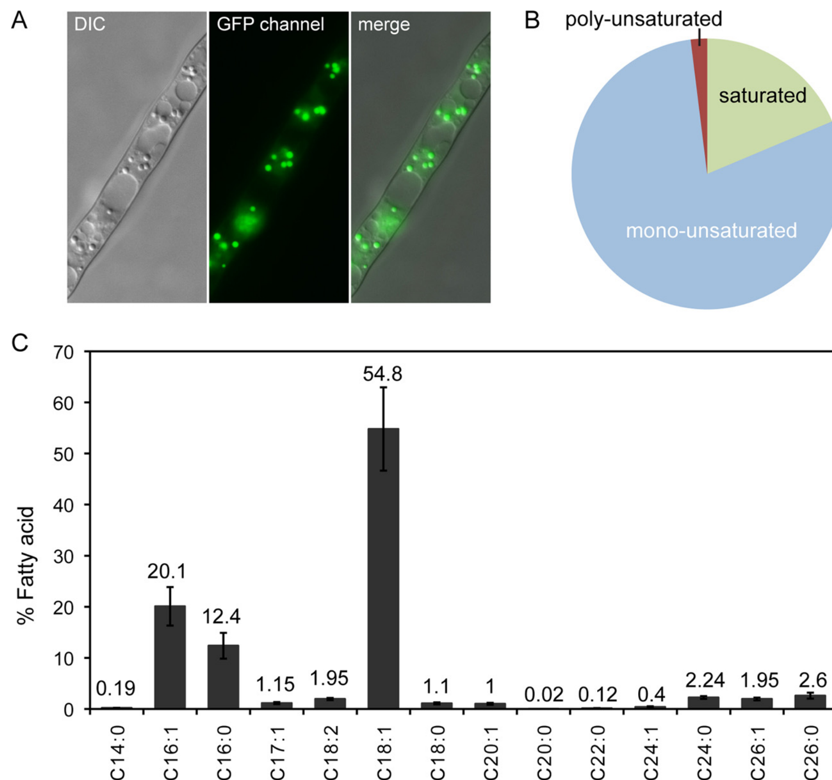

FIG 1 Lipid profile of a wild-type strain of *A. gossypii*. (A) Lipid bodies inside the *A. gossypii* mycelia were stained with Bodipy and visualized under fluorescence microscopy. DIC, differential interference contrast. (B) FA content of a WT strain of *A. gossypii* grown in 8% glucose. (C) Characterization of the FA composition of a WT strain of *A. gossypii* grown in 8% glucose; the numbers indicate the percentage of each FA. The results are the means of two independent experiments performed in duplicate. The error bars represent the standard deviations.

RNA extraction and quantitative real-time PCR. Previously frozen *A. gossypii* mycelium (200 to 300 mg) was homogenized mechanically in liquid nitrogen using TRIzol reagent (Invitrogen, Carlsbad, CA), and total RNA was isolated as described by the manufacturer. RNA was incubated with 20 U of RNase-free DNase I (Roche, Basel, Switzerland). The cDNA samples were prepared using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative real-time PCR was performed with a LightCycler 480 real-time PCR instrument (Roche), using SYBR green I master mix (Roche) following the manufacturer's instructions. Primer sequences are listed in Table S2 in the supplemental material. All real-time PCRs were performed in duplicate and in at least two independent experiments. Quantitative analyses were carried out using the LightCycler 480 software.

Fluorescence microscopy. For the visualization of lipid bodies, *A. gossypii* mycelium was suspended and washed in SB solution. The Bodipy lipid probe (2.5 mg/ml in ethanol; Invitrogen) was added to the hyphal suspension, and the solution was washed three times with SB solution. The lipid bodies were visualized using an Axio Imager.M2 (Zeiss, Le Pecq, France) fluorescence microscope at 495 nm (green fluorescent protein [GFP] filter) with a 100× oil immersion objective. AxioVision release 4.8 software was used to record the images.

RESULTS

Accumulation of FA in *A. gossypii*. *A. gossypii* is considered to be a model organism for the biotechnological production of vitamins, nucleotides, and proteins (19, 29, 30). Furthermore, it has been reported that *A. gossypii* is able to accumulate a significant amount of lipid bodies in its multinucleated hyphae (21) (Fig. 1A). However, *A. gossypii* has typically been regarded as a nonoleaginous microorganism (9).

In order to characterize the lipidogenic (FA) profile of *A. gossypii*, we first wished to determine the total FA content of a wild-type strain of *A. gossypii* grown in medium containing glucose as the sole carbon source. Our results revealed that *A. gossypii* mostly accumulated unsaturated fatty acids, which comprised 81% of the total FA content (Fig. 1B). In good agreement with published results (21), we found that oleic acid ($C_{18:1}$) was the most abundant FA in *A. gossypii* (54.8%), followed to a lesser extent by palmitoleic acid ($C_{16:1}$) and palmitic acid ($C_{16:0}$), which represented 20.1% and 12.4% of total FAs, respectively (Fig. 1C). In addition, we quantified significant levels (representing more than 1% of the total FA content) of other long-chain and very long-chain FAs, including the uncommon FA ginkgolic acid ($C_{17:1}$), and we also found trace amounts (less than 0.5% of the total FA content) of myristic ($C_{14:0}$), arachidic ($C_{20:0}$), behenic ($C_{22:0}$), and nervonic ($C_{24:1}$) acids (Fig. 1C).

Lipid accumulation in *A. gossypii* mutants lacking the beta-oxidation pathway. An increase in lipid accumulation has been achieved previously in engineered microorganisms lacking the FA beta-oxidation pathway, which is the main metabolic pathway that competes with lipid accumulation (10, 14, 15, 22). In contrast to other microorganisms, *A. gossypii* has only one beta-oxidation pathway localized to the peroxisome (9). According to the Ashbya Genome Database (http://agd.vital-it.ch/index.html), the genes *AER358C* (*POX1*), *AGL060W* (*FOX2*), and *AFR302W* (*FOX3*) are syntenic homologs of the *S. cerevisiae* genes *POX1*, *FOX2*, and *POT1*, respectively, which encode the enzymatic activities of the beta-oxidation pathway (Fig. 2A).

In order to evaluate the effect of blocking the beta-oxidation

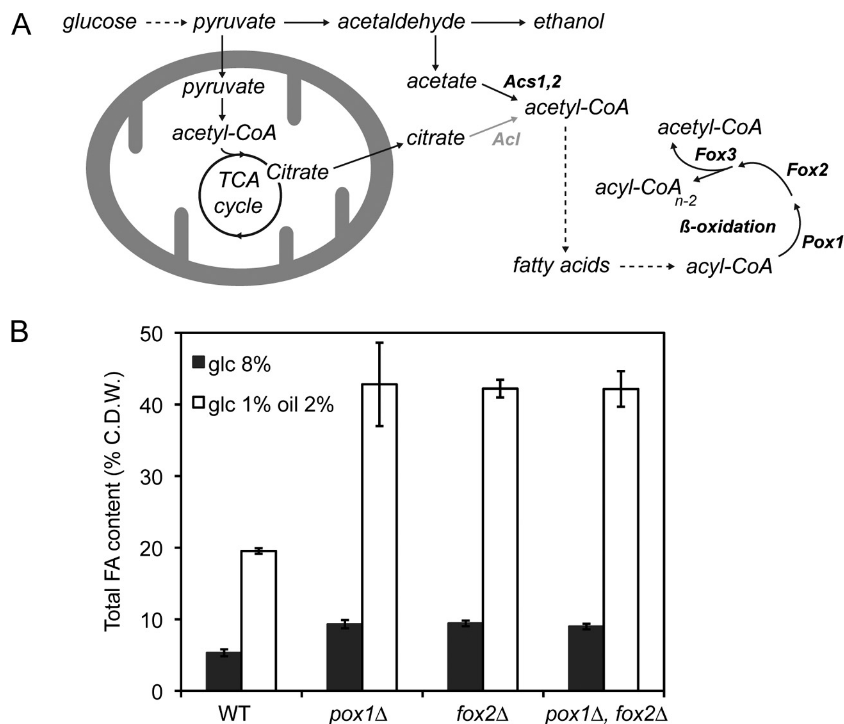

FIG 2 Abolition of the FA beta-oxidation pathway increases lipid accumulation in *A. gossypii*. (A) Simplified diagram of FA biosynthesis and degradation in *A. gossypii*. ACL activity (gray) is not present in *A. gossypii*. The dashed arrows indicate a multistep pathway. (B) Total FA quantification of *A. gossypii* mutant strains lacking beta-oxidation genes. Analyses were carried out with cultures grown in 8% glucose (glc) or 1% glucose plus 2% oleic acid for 3 days. The results are the means of two independent experiments performed in duplicate. The error bars represent the standard deviations. C.D.W., cell dry weight.

process on the accumulation of lipids in *A. gossypii*, we carried out single and double deletions of both the *AER358C* (*POX1*) and *AGL060W* (*FOX2*) genes, which code for acyl-CoA oxidase and the multifunctional beta-oxidation enzyme, respectively. The *pox1*Δ and *fox2*Δ single mutants and the *pox1*Δ *fox2*Δ double mutant did not show any growth defect in glucose-containing medium; however, their growth was impaired when oleic acid was used as the sole carbon source (data not shown). Accordingly, we measured the lipid accumulation of the mutants in glucose-containing medium. As shown in Fig. 2B, abolition of the beta-oxidation pathway triggered a 2-fold increase (up to 9.4% of cell dry weight) in the accumulation of lipids in 8% glucose-containing medium. Furthermore, when oleic acid (2%) was added to the culture medium, the FA content rose to 19.5% of cell dry weight in the WT strain, and after 3 days of culture, the three beta-oxidation mutants displayed a level of lipid accumulation of about 40% of their cell dry weight.

Heterologous expression of *Y. lipolytica ACL* genes in *A. gossypii*. The immediate precursor for the biosynthesis of FA is cytosolic acetyl-CoA, which can be synthesized from both acetate and citrate through two enzymatic pathways (Fig. 2A): the ACS (from acetate) and the ACL (from mitochondrial citrate) pathways (9, 31). In this regard, a correlation between lipid accumulation in oleaginous yeasts and the presence of ACL activity has been reported (32).

The ACL enzyme of plants and most yeasts and fungi is a heteromeric protein that comprises different subunits encoded by the *ACL2* and *ACL1* genes. However, animals and other fungi, such as zygomycetes and basidiomycetes, have homomeric ACL enzymes consisting of a large subunit encoded by a single *ACL* gene, which was originated by gene fusion of *ACL2* and *ACL1* (Fig. 3A) (31, 33). Analysis of the *A. gossypii* genome did not retrieve any predicted ACL-coding gene; accordingly, *A. gossypii* must generate acetyl-CoA for FA biosynthesis through ACS activity (Fig. 2A), as has also been described for *Saccharomyces cerevisiae* (34). In contrast, *Y. lipolytica* contains the *ACL2* and *ACL1* genes encoding the two subunits of the heteromeric ACL enzyme (33). Therefore, we decided to investigate the effect of the heterologous expression of both the *ACL2* and *ACL1* genes from *Y. lipolytica* on the accumulation of FA in *A. gossypii*.

The *ACL2* and *ACL1* ORFs were amplified from *Y. lipolytica* genomic DNA and used to generate two integrative cassettes for their heterologous overexpression in *A. gossypii* under the control of the strong promoter p*AgGPD* (see Materials and Methods). We carried out all the different combinations for the expression of the *Y. lipolytica ACL2* and *ACL1* genes in both the WT and *pox1*Δ backgrounds of *A. gossypii*. The expression cassette for *ACL2* was targeted to the *STE12* locus, while the expression cassette for *ACL1* was inserted into the *LEU2* locus. Integration of the overexpression cassettes in the target genomic locations was confirmed by analytical PCR, and the mRNA levels of both the *ACL2* and *ACL1* genes in the engineered strains of *A. gossypii* were determined by quantitative PCR (Fig. 3B). Next, we measured the acetyl-CoA contents in the mutant strains that overexpressed the two subunits of the ACL enzyme. As shown in Fig. 3C, overexpression of the *ACL* genes induced a strong increase in the acetyl-CoA pool in both the WT and *pox1*Δ backgrounds of *A. gossypii*. Furthermore, we found that the acetyl-CoA content was significantly higher in the *pox1*Δ background than in the WT background (Fig. 3C).

The *ACL*-expressing mutants were cultured in 8% glucose-

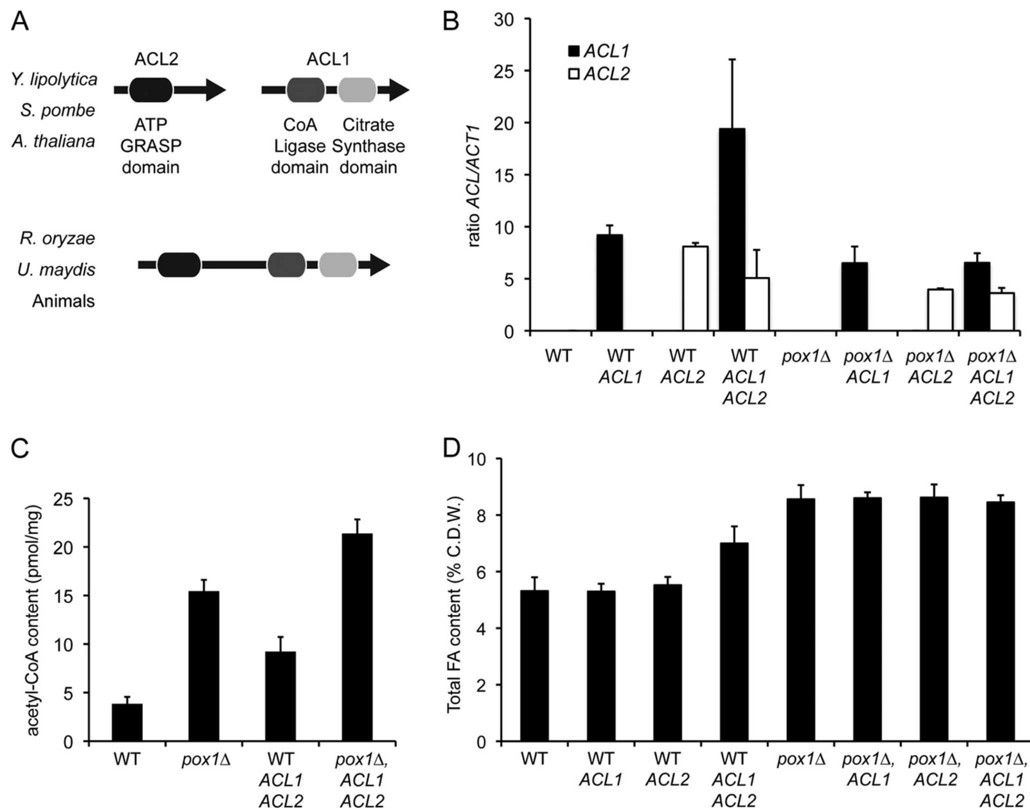

FIG 3 Heterologous expression of the *Y. lipolytica ACL* genes in *A. gossypii*. (A) Domain organizations of ACL-encoding genes in different species. *S. pombe*, *Schizosaccharomyces pombe*; *A. thaliana*, *Arabidopsis thaliana*; *R. oryzae*, *Rhizopus oryzae*; *U. maydis*, *Ustilago maydis*. (B) Relative transcription levels of the *ACL2* and *ACL1* genes in our *A. gossypii* mutant strains. Transcription levels were normalized using the *A. gossypii ACT1* gene as a reference. Relative quantitative analyses were performed using LightCycler 480 software. The results are the means of two independent experiments performed in duplicate and are expressed as a ratio of the cDNA abundances of the target genes with respect to the *ACT1* mRNA levels. (C) Acetyl-CoA quantification in *A. gossypii* strains. The results are the means of two independent experiments performed in duplicate. (D) Total FA quantification in *A. gossypii* strains. Analyses were carried out with cultures grown in 8% glucose for 3 days. The results are the means of two independent experiments performed in duplicate. The error bars represent the standard deviations.

containing medium, and lipid accumulation was determined as described in Materials and Methods. We detected an increase in the lipid content in the strain that overexpressed both the *ACL2* and *ACL1* genes in the WT background (Fig. 3D). However, the increased levels of acetyl-CoA in the *pox*Δ mutant that overexpressed the *ACL* genes did not correlate with any significant change in the accumulation of lipids (Fig. 3D).

Engineered strains of *A. gossypii* as novel SCOs. As mentioned above, *A. gossypii* has been considered a nonoleaginous organism on the basis of two principal features: (i) a lipid accumulation capacity below 20% of the total cell dry weight and (ii) the absence of ACL-coding genes. However, we observed that the lipid content of a *pox1*Δ mutant of *A. gossypii* was able to exceed 40% of the mycelial dry weight when oleic acid was present in the culture medium (Fig. 2B).

A. gossypii shows two differentiated growth stages that strongly affect the production of metabolites such as riboflavin: a trophic phase, when the growth rate increases exponentially and riboflavin production is minimal, and a productive phase, when the growth rate decreases and riboflavin is overproduced (19). Accordingly, we wished to check whether the accumulation of lipids in our engineered strains changed during both the trophic and the productive phases. We measured the lipid contents at different time points of both the trophic and productive phases during 7 days of culture in oleic acid-containing media (Fig. 4A) and observed that the accumulation of lipids was maximal in the *pox1*Δ strain at the end of the productive phase, yielding a total FA content of more than 70% of cell dry weight. To our knowledge, this represents one of the highest lipid accumulation rates described so far (4, 7, 8). However, we also found that overexpression of both the *ACL2* and *ACL1* genes from *Y. lipolytica* induced an increase in the FA content in a WT strain only during the trophic phase (after 3 days of culture). In addition, during the productive phase (after 7 days of culture), the expression of ACL activity did not change the accumulation of lipids or even induce a decrease in the FA content in the WT and the *pox1*Δ strains, respectively (Fig. 4A). As shown in Fig. 4B, the accumulation of lipids in the *pox1*Δ strain could be visualized directly by staining of the intracellular lipid bodies, which were significantly larger in the *pox1*Δ strain than in the wild type.

DISCUSSION

The development of novel microbial factories with the ability to transform low-value lipids into different fine chemicals of industrial significance is an important issue for biotechnology. The presence of lipid bodies in *A. gossypii* hyphae has been reported previously (21); however, the biotechnological potential of *A. gossypii* for SCO production has remained unexplored. Here, we analyzed the FA content of a wild-type strain of *A. gossypii*, and we

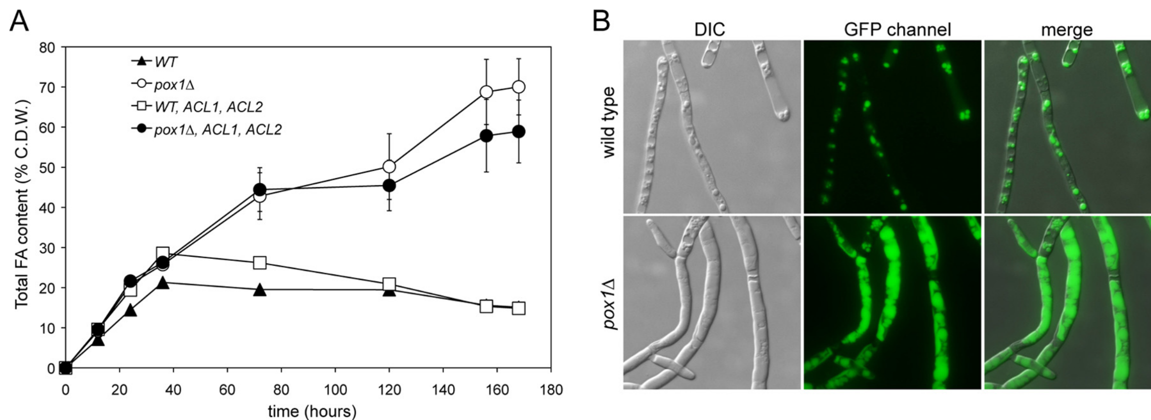

FIG 4 Lipid accumulation in the engineered *A. gossypii* strains. (A) Lipid quantification in *A. gossypii* strains at different time points of cultures grown in 1% glucose plus 2% oleic acid at 0 to ~50 h (trophic phase) and 50 to 168 h (productive phase). The results are the means of two independent experiments performed in duplicate. The error bars represent the standard deviations. (B) Micrographs of the WT and *pox1*Δ strains of A. *gossypii* grown in 1% glucose plus 2% oleic acid for 7 days. Lipid bodies were stained with Bodipy and visualized under fluorescence microscopy.

found that unsaturated FAs represented more than 80% of the total FA composition, suggesting an effective system of FA desaturation in *A. gossypii*. Indeed, a BLAST search for *A. gossypii* orthologs of the *OLE1* gene, which codes for a delta-9 desaturase in *S. cerevisiae* (35), retrieved two different ORFs (*AAL078W* and *AAR153C*) that were predicted to encode desaturases. We also identified significant amounts of C_{24} and C_{26} FAs, which indicates the presence of enzymatic activities that catalyze the elongation of the acyl-CoA chain toward the synthesis of very long-chain FAs in *A. gossypii*.

According to general criteria regarding biomass, an oleaginous microorganism must be able to accumulate a minimum lipid content of 20% (4, 7, 8). Also, it has been proposed that lipid accumulation mostly depends on a continuous supply of both cytosolic acetyl-CoA and NADPH as the reducing power for FA biosynthesis (8), and by chance, two enzymes have been suggested to be essential for these metabolic processes, namely, ATP-citrate lyase and malic enzyme (8, 36). In this regard, it has been reported that the presence of an active ACL enzyme is a general feature of all oleaginous species (32, 36), although we observed that a wild-type strain of *A. gossypii* that lacks ACL-coding genes was able to accumulate lipids at a high concentration (19.5% of dry biomass), although still below the level of an oleaginous microorganism. However, when the ACL enzyme from *Y. lipolytica* was overexpressed in *A. gossypii*, the total FA yield reached 26% of the cell dry weight in the early phases of growth, indicating that *ACL* genes are likely essential for the lipidogenic ability of oleaginous microorganisms. Nevertheless, the presence of ACL activity did not correlate with such an increase in lipid accumulation in a *pox1*Δ mutant of *A. gossypii*, supporting a different hypothesis. First, the *pox1*Δ mutant must undergo metabolic flux redirection that triggers an increase in the cytosolic acetyl-CoA pool independently of ACL activity. Indeed, alternative pathways derived from carbohydrate and amino acid metabolism have also been described for the synthesis of acetyl-CoA (9). Second, a high concentration of acetyl-CoA in the *pox1*Δ *ACL2 ACL1* strain could induce pleiotropic effects that would mask the effect of the overexpression of the ACL enzyme on lipid accumulation in the *pox1*Δ background. For example, the *pox1*Δ *ACL2 ACL1* strain might undergo a depletion of the coenzyme A pool induced by the increased ACL activity that finally triggers inhibition of the activation of extracellular FAs. In addition, the nucleocytosolic pool of acetyl-CoA is also used for histone acetylation, which is crucial for the epigenetic mechanisms of transcriptional regulation (37). Therefore, the ACS activity and other metabolic reactions that provide cytosolic acetyl-CoA must be regulated to avoid drawbacks in the global gene expression pattern. In any case, the high level of acetyl-CoA in our engineered strains might serve for future biotechnological approaches involving the manipulation of acetyl-CoA metabolism, such as the production of biobutanol (38).

We also found a significant difference in the lipid accumulation pattern between the WT and *pox1*Δ backgrounds during the trophic and productive phases of *A. gossypii* growth. Indeed, as shown in Fig. 4A, the *pox1*Δ mutants increased lipid accumulation after 3 days of culture and throughout the productive phase. In contrast, the *POX1* strains (WT backgrounds) underwent a decrease in the FA content after the trophic phase. This difference is probably due to activation of the beta-oxidation pathway during the productive phase.

Metabolic engineering for SCO production has been carried out in many microorganisms using different approaches that generally involve either the overexpression of lipid biosynthesis genes or the inactivation of FA-degrading activities by gene deletion (10, 12). With the aim of increasing the lipid accumulation in *A. gossypii*, we took a multigene approach in *A. gossypii* by exploiting both the overexpression of the *ACL* genes from *Y. lipolytica* and the inactivation of the beta-oxidation pathway by *POX1* gene deletion. As mentioned above, the heterologous expression of *ACL* genes in *A. gossypii* afforded an FA content of 26% of the cell dry weight, thus transforming *A. gossypii* into an oleaginous fungus. Nevertheless, we found that blockade of the beta-oxidation pathway in the *pox1*Δ mutant was the most efficient modification for increasing lipid accumulation in *A. gossypii* up to 70% of the dry biomass, which makes our *pox1*Δ strain an attractive tool for oil biotechnology. The lack of FA degradation in the *pox1*Δ strain in combination with high levels of acetyl-CoA (4-fold more than the wild type) may explain the strong increase in the FA content measured in the strain. Recent studies have described the metabolic engineering of lipid biosynthesis in both oleaginous and nonoleaginous microorganisms, with rates of lipid accumulation

not exceeding 60% of cell dry weight (12, 14, 15, 39, 40). Such modifications include increasing intracellular citrate levels, the overexpression of *ACL* genes, the overexpression of genes to enhance TAG biosynthesis, and the abolition of the beta-oxidation pathway and other competing pathways (12, 14, 15, 22, 23, 40).

A. gossypii is currently used as a microbial factory for several bioprocesses utilizing both low-cost and waste oil to cultivate the fungus (16). Therefore, it is possible to take advantage of the ability of *A. gossypii* to accumulate lipids for its use in biotransformation processes. Further characterization and modification of the *A. gossypii* genes that are predicted to encode desaturases and elongases may allow the design, through metabolic engineering, of new strains that accumulate valuable high-cost oils. This approach to the practical economic use of waste oil is emerging as a sustainable opportunity for the oleochemical industry (5, 41, 42). In this context, *A. gossypii*, which naturally produces mostly unsaturated FAs, may represent a novel tool for the production of high-cost oils, such as PUFAs, considered to be nutraceuticals for food fortification.

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Artículo 5:

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ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Tuning Single-Cell Oil Production in *Ashbya gossypii* by Engineering the Elongation and Desaturation Systems

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ABSTRACT: Microbial oils represent a sustainable alternative to vegetable oils and animal fats as feedstock for both the chemical and biofuel industries. The applications of microbial oils depend on their fatty acid composition, which is defined by the relative amount of each fatty acid, also considering the length and unsaturations of the acyl chain. These two properties are determined by elongases and desaturases. In the present study, we characterized the elongase and desaturase systems in the filamentous fungus *Ashbya gossypii*, which is able to accumulate high amounts of lipids. Additionally, both the elongation and desaturation systems were engineered in order to broaden the potential applications of *A. gossypii* oils. Finally, the properties of the strains engineered for biodiesel production were analyzed, with the observation that *A. gossypii* is a good candidate for the microbial production of renewable biofuels.

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KEYWORDS: *Ashbya gossypii*; single-cell oil; elongase; desaturase; biodiesel

Introduction

Biodiesel is usually produced by transesterification of vegetable oils or animal fats. However, their massive use has generated environmental and economical concerns. Thus, the use of non-edible oil sources such as microbial oils (Single-Cell Oils, SCOs) represents a more sustainable choice for the production of biodiesel (Kosa and Ragauskas, 2011; Yu et al., 2013). Additionally, SCOs can be used for the production of other carbon-based compounds such as lubricants, functional polymers and other high-value fine chemicals and nutraceuticals (Beopoulos et al., 2011; Buijs et al., 2013; Kocharin et al., 2013).

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SCOs have several advantages over other oil resources since their production is not affected either by climate or seasonal conditions. In addition, oleaginous microorganisms are able to grow using a wide range of inexpensive carbon supplies, including certain waste carbon sources. Additionally, they have short life cycles with rapid growth rates and they can be grown in bioreactors. Consequently, microbial fermentations can readily be scaled up in order to accommodate production to market needs (Liang and Jiang, 2013).

Ashbya gossypii is a filamentous hemiascomycete that has been extensively used for the microbial production of riboflavin (Jiménez et al., 2005, 2008; Ledesma-Amaro et al., 2013; Mateos et al., 2006). In addition, we have recently shown that *A. gossypii* is able to accumulate up to 70% of the cell dry weight as fatty acids (Ledesma-Amaro et al., 2014). Moreover, *A. gossypii* is able to grow in low-cost oils, which makes this fungus a good candidate for SCO production by metabolic engineering. The use of *A. gossypii* for microbial fermentation presents other biotechnological advantages, such as the autolysis of its hyphae and harvesting of the mycelia by simple filtration. Moreover, *A. gossypii* can be grown by pellet fermentation, which reduces broth viscosity and improves mixing and mass transfer, saving both costs and energy, as described for other filamentous fungi (Zheng et al., 2012).

The properties and applicability of SCOs mostly depend on their lipid composition, which is characterized by the relative amount of each fatty acid, also defined by the acyl chain-length and the number of double bonds. There are two main enzymatic activities that determine the length and unsaturations of FAs: elongases and desaturases, respectively (Hashimoto et al., 2008). They both operate downstream of FAS (fatty acid synthase), which usually catalyzes the synthesis of saturated FAs up to 16 or 18 carbons in length (Denic and Weissman, 2007).

Fatty acid elongation occurs in a series of four enzymatic reactions that are catalyzed by β-ketoacyl-CoA synthase, β-ketoacyl-CoA reductase, β-hydroyl-CoA dehydratase, and

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enoylCoA reductase (Denic and Weissman, 2007). The elongases catalyze a condensation reaction, which adds two carbons to the fatty acid backbone in each cycle. The chain-length of both the substrate and the product of elongases depend on the specificity of each enzyme. For example, in *S. cerevisiae* there are three genes encoding elongases with different specificities for the chain-length of the substrates and products: *ELO1, FEN1*, and *SUR4* (Oh et al., 1997; Toke and Martin, 1996).

Desaturases are enzymes that catalyze the incorporation of a double bond into an acyl chain with strict regioselectivity and stereoselectivity. Four subfamilies of desaturases have been defined functionally: (i) the First Desaturase subfamily, which introduces the first double bond into a saturated FA, (ii) the Omega Desaturase subfamily, which catalyzes a double bond incorporation between a pre-existing double bond and the end of the FA, (iii) the Front-End Desaturase subfamily, which introduces the double bond between a pre-existing double bond and the carboxyl end of the acyl chain and (iv) the sphingolipid Desaturase subfamily, which comprises sphingolipid $\Delta 4$ desaturases (Hashimoto et al., 2008).

Modification of the elongation and desaturation systems by means of metabolic engineering is intended to improve the FA profile of SCOs for the production of certain specific high-value compounds. For example, the production of omega-3 FAs such as eicosapentaenoic acid (EPA) has been achieved by heterologous expression of both elongases and desaturases (Xue et al., 2013). In addition, one of the most promising applications of microbial oils could be the production of biodiesel (Caspeta and Nielsen, 2013). Fatty acid methyl esters (FAMEs) of SCOs can be used directly as biodiesel. However, their quality is strongly influenced by the FA composition of SCOs. Accordingly, metabolic engineering must be applied for the optimization of the FA composition of SCOs for biodiesel applications.

Here, we studied the elongation and desaturation systems of *A. gossypii*. We carried out metabolic engineering of both the elongation and desaturation systems to modify the FA profile of *A. gossypii*. The industrial significance of these modifications is discussed and the application of SCOs from *A. gossypii* for biodiesel production is further analyzed.

Materials and Methods

Strains, Media, and Techniques for *A. gossypii* and *S. cerevisiae* Culture

The *A. gossypii* ATCC 10895 strain was used and was considered a wild-type strain. *A. gossypii* was cultured either in MA2 rich medium (Mateos et al., 2006) or SO medium (2% bactopeptone, 0.2% yeast extract, 1% soybean oil, 0,06% myo-inositol) at 28°C. A concentration of 250 μg/mL of Geneticin (G418) (Sigma, Steinheim, Germany) or 100 μg/mL of clonNAT (Werner Bioagents) was used where indicated. *A. gossypii* transformation, sporulation conditions and spore isolation were as previously described (Mateos et al., 2006). RNA isolation was carried out as described elsewhere (Ledesma-Amaro et al., 2014).

The *S. cerevisiae* strains used in this study were BY4741, *elo1Δ, fen1Δ, and sur4Δ* (Euroscarf; Frankfurt, Germany). Cells were routinely grown at 28°C in synthetic complete (SC) medium lacking either uracil or leucine (SC-Ura or SC-Leu) and containing either 2% glucose or 2% galactose plus 1% raffinose as carbon sources. *S. cerevisiae* transformation was carried out following the lithium acetate method.

For the supplementation of culture media with fatty acids (250 μM), 1% tergitol™ solution (Sigma) was also added to the media.

PCR-Based Cloning of the *AFR589C, AFL079W, AFR624W* and *AFR586W* Genes From *A. gossypii*

The annotated sequences of *AFR589C*, *AFL079W*, *AFR624W*, and *AFR586W* in the Ashbya Genome Database (AGD; http://agd.vital-it.ch/index.html) were used to design pairs of primers for each gene (Table SI). The ORFs of *AFR589C*, *AFL079W*, *AFR624W*, and *AFR586W* were amplified by PCR using *A. gossypii* genomic DNA. The PCR amplicons were cloned into the pGEM-T Easy vector (Promega). All four ORFs were confirmed by sequencing of the entire DNA fragments (data not shown).

Heterologous Expression of the *AFR589C, AFL079W, AFR624W* and *AFR586W* Genes in *S. cerevisiae*

The ORFs encoding predicted *A. gossypii* elongases and desaturases were PCR-amplified to add specific restriction-site ends using the primers listed in the Table SI. The ORFs corresponding to *AFR624W* and *AFL079W* were cloned between the *Bam*HI and *Sal*I restriction-sites of the yeast expression vector pESC-LEU (Stratagene) under the control of the *GAL1* promoter. The *AFR589C* and *AFR586W* ORFs were cloned as *Not*I–*Eco*RI fragments into the pESC-URA expression vector (Stratagene) under the control of the *GAL10* promoter. The *S. cerevisiae* strains indicated were transformed with the above plasmids and the resulting transformants were selected for either leucin or uracil prototrophy. For gene expression the *S. cerevisiae* strains were grown in glucose-containing medium until the cultures reached an OD600 of 0.5. Then, the cells were washed and grown in 2% galactose plus 1% raffinose-containing medium during 48 h before lipid analysis.

Gene Deletion and Overexpression of the *AFR589C, AFL079W, AFR624W* and *AFR586W* Genes in *A. gossypii*

For gene overexpression, the promoter sequence of the *AgGPD* gene was integrated upstream of the ATG initiator codon of each gene. An overexpression cassette comprising the *AgGPDp* and the *KanMX6* selectable marker, conferring resistance to geneticin (G418), was PCR-amplified using specific primers for each gene (Table SI). The overexpression modules were

used to transform spores of *A. gossypii* and positive clones were selected in G418-containing medium. Homokaryon clones were obtained by sporulation of the primary transformants. The correct genomic integration of each overexpression cassette was confirmed by analytical PCR followed by DNA sequencing (data not shown). Gene overexpression was checked by qRT-PCR analysis (data not shown).

For gene deletion, a gene-replacement cassette was constructed for each gene by PCR amplification of the *KanMX6* marker (see primers in the Table SI). The replacement cassettes were used to transform spores of *A. gossypii* and primary transformants were selected in G418-containing medium. Homokaryon clones were isolated by sporulation of the primary transformants. The homologous integrations of the selection markers were confirmed by analytical PCR followed by DNA sequencing (data not shown).

Fatty Acid Analysis

Fatty acid methyl esters (FAME) were obtained by transmethylation of the freeze-dried *A. gossypii* biomass with BF_3 12% (v/v) in methanol. 100 μg of mycelia was mixed with 1 mL of hexane and 750 μL of BF_3-Methanol under a nitrogen atmosphere. The mixture was incubated for 45 min at 100°C. The samples were cooled on ice and the reaction was stopped by the addition of 0.5 mL of distilled water. The upper phase was recovered after centrifugation and the extraction step was repeated by adding 0.5 mL of hexane to the lower phase. The two hexane-soluble phases were mixed and evaporated with nitrogen. The FAMEs were resuspended in 100 μL of hexane and used for GC-MS analysis. GC-MS was carried out in a Shimadzu QP5000 mass spectrometer with a Shimadzu GC17 gas chromatograph. Conditions for analysis were as follows: Column DB-5 30 m, 0.25-mm diameter, and 0.25-micron film. Helium was used as the carrier gas at 1.3 mL/min with a split ratio of 60:1. The injector temperature was 270°C and the interface temperature was 290°C. The oven program was as follows: initial temperature 90°C for 5 min, followed by a ramp of 12°C/min up to 190°C, a ramp of 4°C/min up to 290°C, holding for 5 min. The identification of the compounds was performed according to the NIST98 database (http://www.nist.gov/).

Quantitative Real-Time PCR

Previously frozen mycelium of *A. gossypii* (200–300 mg) was homogenized using TRIzol reagent (Invitrogen, Carlsbad, CA) and total RNA was isolated. Template cDNA for quantitative real-time PCR was synthesized using 1 μg of total RNA using oligo-dT primer (Transcription First Strand cDNA Synthesis Kit, Roche). Primer pairs were designed for *AFR589C*, *AFL079W*, *AFR624W*, and *AFR586W* and the gene *ACT1* (see primer list, Table SI). 3 μL of cDNA was used for the qRT-PCR reaction (Light Cycler® 480 SYBR Green I Master, Roche) in a Light Cycler™ 480 device (Roche). All experiments were carried out in triplicate and analyzed with Light Cycler™ 480 software version 1.5 (Roche).

Determination of Biodiesel Properties

Mathematical equations and predictive models were used to theoretically determine all the physicochemical fuel-related parameters of the *A. gossypii* SCOs (Garcia et al., 2010; Khot et al., 2012).

Density values at 15°C were calculated with a previously described (Pratas et al., 2011) predictive model (Equation 1). The density of individual FAMEs was used for calculation (Lapuerta et al., 2010).

$$\rho = \sum C_i \times \rho_i \tag{1}$$

where C_i is the mass fraction of component i, and ρ_i is the density of the individual component present in the fuel mixture.

Kinematic viscosities at 40°C were estimated by Equation 2, which is a simplification of the Grunberg–Nissan equation (Knothe and Steidley, 2011). In this algorithm, the viscosity values are applied directly instead of their logarithms.

$$\nu_{\mathrm{mix}} = \sum A_C \times \nu_C \tag{2}$$

where ν_{mix} is the kinematic viscosity of the biodiesel sample; A_C is the relative amount (%/100) of the individual neat ester in the mixture (as determined by gas chromatography), and ν_C is the kinematic viscosity of those individual esters.

The Iodine Value (IV) and Saponification Number (SN) were calculated empirically using the mixing rule (Equations 3 and 4) and individual FAME data (Demirba , 1998; Gopinath et al., 2009; Krisnangkura, 1991).

$$\mathrm{IV}_{\mathrm{mix}} = \sum A_C \times \mathrm{IV}_C; \tag{3}$$

$$\mathrm{SN}_{\mathrm{mix}} = \sum A_C \times \mathrm{SN}_C \tag{4}$$

where $\mathrm{IV/SN}_{\mathrm{mix}}$ is the IV/SN of the mixture; A_C is the relative amount (%/100) of the individual ester in the mixture; $\mathrm{IV_C}$ is the IV of the individual esters, and $\mathrm{SN_C}$ is the SN of the individual esters.

The higher heating value (HHV) was predicted using Equation 5, where the HHV depends on the IV and SN of that fuel (Demirba , 1998).

$$\mathrm{HHV} = 49.43 - 0.041 \times \mathrm{SN} - 0.015 \times \mathrm{IV} \tag{5}$$

The Cetane Number (CN) was calculated empirically using a multiple regression equation (Equation 6) (Tong et al., 2011).

$$\mathrm{CN} = 1.068 \sum (\mathrm{CNi} \times \mathrm{Wi}) - 6.747 \tag{6}$$

where CN represents the cetane number of the final mixture; Wi is the mass fraction of individual FAME, and CNi is the

CN of the pure FAME calculated with Equations 7 and 8 (Tong et al., 2011).

$$\text{CNi(unsaturated fatty acids)} = 109.000 - 9.292\text{X} + 0.354\text{X}^2 \quad (7)$$

$$\text{CNi(saturated fatty acids)} = 107.71 + 31.126\text{X} - 2,042\text{X}^2 + 0.0499\text{X}^3 \quad (8)$$

where X is the carbon number of each individual fatty acid.

Cold Filter Plugging Poing (CFPP) was calculated using the Long-Chain Saturated Factor (LCSF) as previously described (Ramos et al., 2009). The relationship between CFPP and LCSF can be observed in Equation 9.

$$\text{CFPP} = 3.1417 \times \text{LCSF} - 16.477 \quad (9)$$

Results and Discussion

Identification of Putative Elongases and Desaturases from *A. gossypii*

The elongation and desaturation systems from *A. gossypii* have been identified previously in an in silico analysis (Hashimoto et al., 2008); however, there are no experimental data that functionally characterize, and hence confirm, the identification of the FA elongation and desaturation systems of *A. gossypii*, which determine the total FA composition to a substantial extent.

The sequences of the predicted elongases (*AFR586W* and *AFR624W*) and desaturases (*AFL079W*, *AFR589C*, *AAR153C*, *AAL078W*, and *AGR025W*) of *A. gossypii* were obtained from the KEGG Genes database (http://www.kegg.jp/dbget-bin/www_bfind?genes). CLUSTALW analysis confirmed their homology with elongases and desaturases from evolutionarily related species such as *S. cerevisiae*, *Yarrowia lipolytica*, and *Kluyveromyces lactis* (Fig. 1).

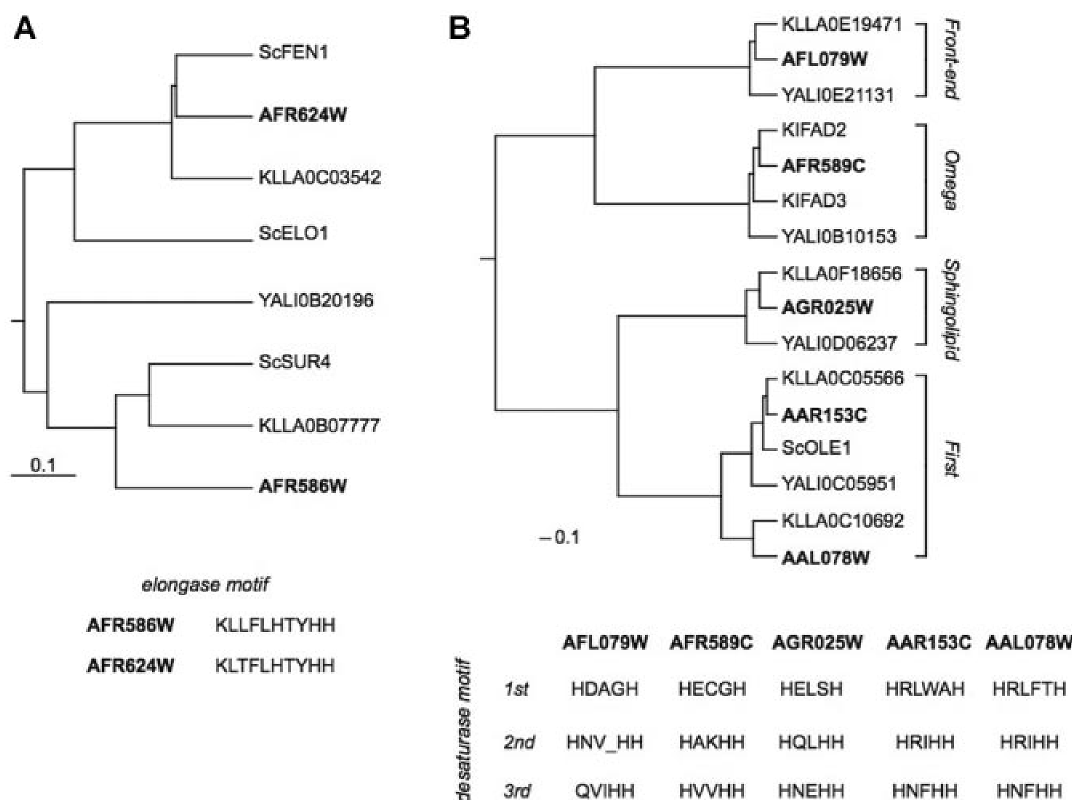

Figure 1. Predicted elongases and desaturases from *A. gossypii*. **A**: Phylogenetic tree of elongases. The elongase motifs in the *A. gossypii* proteins are shown below. **B**: Phylogenetic tree of desaturases. The three desaturase motifs in the *A. gossypii* proteins are shown below. Sequences from *A. gossypii* (bold), *S. cerevisiae*, *Y. lipolytica*, and *K. lactis* were used for a ClustalW alignment (http://www.genome.jp/tools/clustalw/) and a rooted phylogenetic tree using the UPGMA method was constructed. Accession numbers for elongases are: NP_009963 (ScFEN1); NP_986171 (AFR624W); XP_452354 (KLLA0C03542); NP_012339 (ScELO1); XP_501125 (YALI0B20196); NP_013476 (ScSUR4); XP_451876 (KLLA0B07777); NP_986133 (AFR586W). Accession numbers for desaturases are: CAG99919 (KLLA0E19471); AAS53293 (AFL079W); XP_504218 (YALI0E21131); CAG98110 (KIFAD2); AAS53960 (AFR589C); CAH01944 (KIFAD3); XP_500707 (YALI0B10153); CAG98624 (KLLA0F18656); AAS54514 (AGR025W); XP_502476 (YALI0D06237); CAH01298 (KLLA0C05566); AAS50520 (AAR153C); CAA96757 (ScOLE1); XP_501496 (YALI0C05951); CAH01527 (KLLA0C10692); AAS50288 (AAL078W).

The proteins Afr586wp and Afr624wp showed more than 60% identity with the *S. cerevisiae* elongases *ScSUR4* and *ScFEN1* and both proteins from *A. gossypii* contained the putative consensus sequences of elongases (Fig. 1A). In addition, a homology analysis of the *A. gossypii* proteins Afl079wp, Afr589cp, Aar153cp, Aal078wp, and Agr025wp with desaturases from other microorganisms revealed that the *A. gossypii* proteins can be grouped within the four previously defined subfamilies of desaturases: the First Desaturase (*AAR153C* and *AAL078W*), the Omega Desaturase (*AFR589C*), the Front-End Desaturase (*AFL079W*), and the Sphingolipid Desaturase (*AGR025W*) (Fig. 1B). The five *A. gossypii* proteins analyzed also contained the three typical sequences of the desaturase motif.

Heterologous Expression of *A. gossypii* Elongases and Desaturases in *S. cerevisiae*

We next wished to characterize the function of the *A. gossypii* elongation and desaturation systems. Both the *AAL078W* and *AAR153C* genes (homologs of the *S. cerevisiae* gene *OLE1*) were excluded from the analyses because they have previously been proposed to encode Δ9 desaturases, which determine the presence of oleic acid as the most abundant FA in *A. gossypii* (Stahmann et al., 1994). We also decided to exclude the *AGR025W* gene, which was predicted to encode an sphingolipid desaturase without any essential role in the production of fatty acids with direct industrial applications. Thus, our functional analysis focused on the predicted elongases *AFR586W* and *AFR624W*, and the desaturases *AFR589C* and *AFL079W*.

To demonstrate the functionality of these proteins we carried out their heterologous expression in *S. cerevisiae*, for which the elongation and desaturation systems are well known (Oh et al., 1997; Toke and Martin, 1996). Four different genetic backgrounds of *S. cerevisiae* were used for the overexpression of the predicted elongases *AFR586W* and *AFR624W*: BY4741 (wild-type), *elo1Δ*, *fen1Δ*, and *sur4Δ*. While *ELO1* is involved in medium-chain acyl elongation

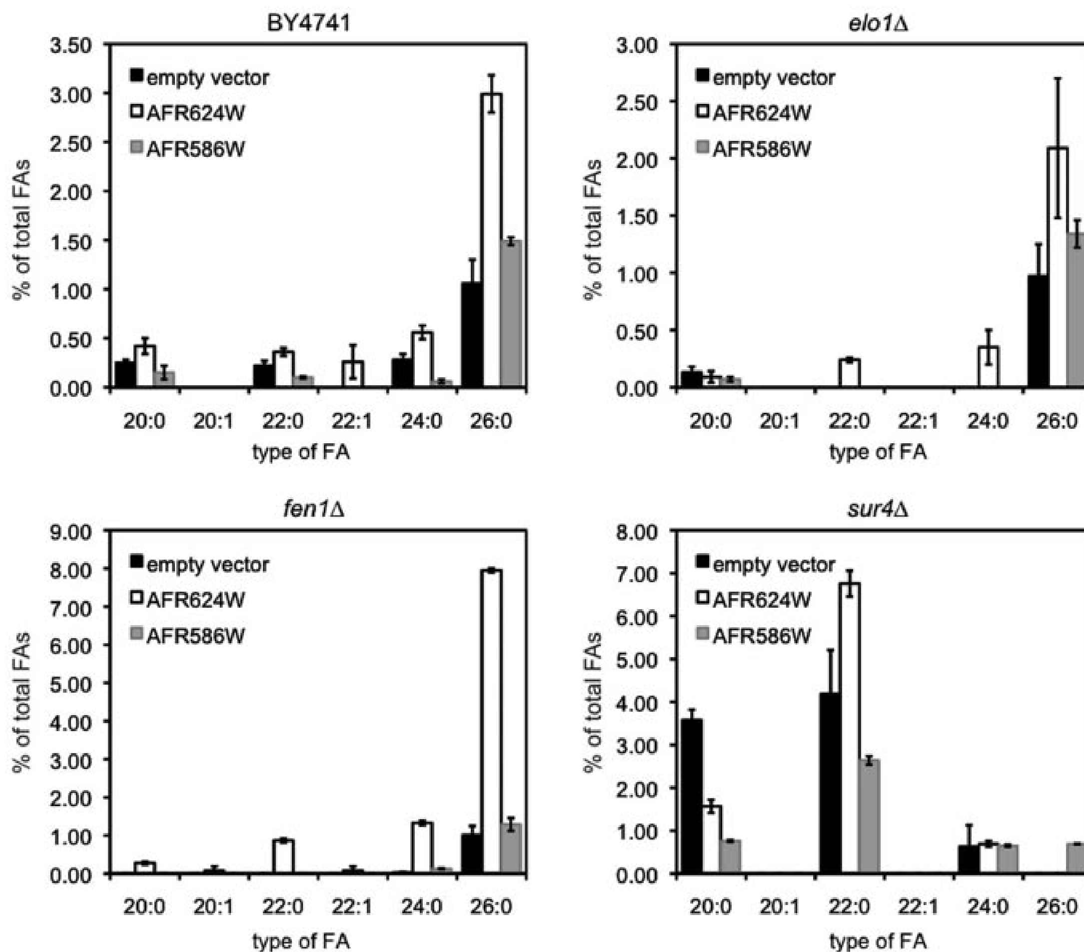

Figure 2. Heterologous expression of predicted *A. gossypii* elongases in *S. cerevisiae*. Profile of very long-chain FAs in four strains of *S. cerevisiae* (BY4741, *elo1*△, *fen1*△, and *sur4*△) that overexpressed either the *AFR624W* gene or the *AFR586W* gene. The strains transformed with the empty vector were used as controls. Data are means of two independent experiments performed in duplicate. Error bars represent standard deviations. Gene expression was achieved in 2% galactose plus 1% raffinose-containing medium during 48 h before lipid analysis.

(from 12:0–16:0 to 16:0–18:0) (Toke and Martin, 1996), *FEN1* and *SUR4* activities catalyze the elongation of long- and very long-chain FAs (up to 24:0 and 26:0, respectively) and, consequently, *SUR4* is also associated with the biosynthesis of sphingolipids (Oh et al., 1997). It was found that 95% of the total FA content of BY4741 corresponded to 16:0, 16:1, 18:0, and 18:1 FAs (Table SII); in contrast, the *elo1Δ* mutant showed an increase in shorter FAs (14:0 and 14:1) and a decrease in the amount of 22:0 and 24:0 FAs (Table SII. In addition, the *fen1Δ* mutant showed the absence of 20:0 and 22:0 FAs and a very low amount of 24:0 FAs, while the *sur4Δ* mutant was characterized by a complete lack of 26:0 FAs and the accumulation of 20:0, 22:0, and 24:0 FAs (Table SII).

The ORFs of the *AFR586W*, *AFR624W*, *AFR589C*, and *AFL079W* genes were PCR-amplified and cloned into an episomal pESC vector for their heterologous overexpression in *S. cerevisiae*.

The expression of both *AFR586W* and *AFR624W* induced significant changes in the FA profiles of the *S. cerevisiae* strains analyzed. AFR624W triggered a general increase in the long-chain and very long-chain FAs (Fig. 2 and Table SII). We defined long-chain FAs as those of 18 carbons, while FAs with longer chains are considered to be very long-chain FAs. The levels of cerotic acid (26:0) were increased after the expression of *AFR624W* in BY4741, *elo1△* and *fen1△*, but not in the *sur4△* strain (Fig. 2 and Table SII), suggesting that *AFR624W* encodes an FA elongase that is able to use 18:0 FAs as substrates to produce longer-chain FAs, but not 26:0 cerotic acid. However, the expression of *AFR586W* also induced an increase in 26:0 FAs in both the BY4741 and *elo1△* strains, but in contrast to *AFR624W* it was able to restore the wild-type phenotype in the *sur4△* strain (Fig. 2 and Table SII), indicating that *AFR586W* likely codes for an FA elongase that produces very long-chain FAs such as cerotic acid (26:0).

The heterologous expression of the predicted desaturases AFR589C and AFL079W was carried out in the BY4741 strain of *S. cerevisiae* and a GC-MS analysis of the yeast strains was performed to determine the total FA composition (Table SII). The expression of the Afl079wp protein did not cause any significant change in the FA profile of the BY4741 strain. In contrast, we identified the presence of linoleic acid (18:2) after the expression of Afr589cp (Table SII), a protein that cluster with other Δ12 desaturases such as YALI0B10153 from *Y. lipolytica* (Fig. 1B) (Beopoulos et al., 2013). Accordingly, Afr589cp may encode a desaturase belonging to the omega desaturase subfamily, which comprises Δ12 desaturases. Indeed, this catalytic activity was further confirmed by adding oleic acid (18:1) to the culture medium and this was efficiently transformed into linoleic acid (18:2) by the Afr589cp-overexpressing strain, as expected (data not shown). Other desaturation substrates were used to identify the activity of the Afl079wp protein: linoleic acid (18:2) was used as a substrate to detect Δ6 desaturase activity but no gamma or alpha linolenic acid was detected in the FA content of any of the strains (data not shown). The Δ8 desaturase activity was also analyzed by the addition of eicosadienoic acid (20:2), but we did not find the product in any of the overexpressing strains. Therefore, the predicted desaturase Afl079wp might not be involved in FA desaturation, but might function as a sphingolipid desaturase. Indeed, the Afl079w protein showed homology with a Δ8 sphingolipid desaturase from *Arabidopsis thaliana* (Sperling et al., 1998), which could well support this hypothesis. In addition, this activity has also been found in *A. gossypii*-related yeasts such as *Saccharomyces kluyveri* and *Kluyveromyces lactis* (Takakuwa et al., 2002).

Transcriptional Regulation of *A. gossypii* Elongases and Desaturases

The transcription of elongases and desaturases can be regulated by the carbon source. Accordingly, a switch from the de novo synthesis of FAs to the direct uptake of FAs from the medium might occur. In light of this, we were prompted to evaluate the transcriptional regulation of the *AFR586W*, *AFR624W*, *AFR589C*, and *AFL079W* genes from *A. gossypii*. We determined the relative transcriptional activity of each gene in cultures of the wild-type strain grown in two different media: MA2 (rich media with glucose as carbon source) and SO (rich media with soybean oil as carbon source). We found that the transcription of both *AFR586W* and *AFR624W* was positively regulated by the presence of soybean oil as the sole carbon source (Fig. 3). Soybean oil contains 99.5% of mid- and long-chain FAs (≤18C), which constitute the main substrates of elongases. In addition, the transcription of the *AFR589C* gene appeared to be negatively regulated (with a

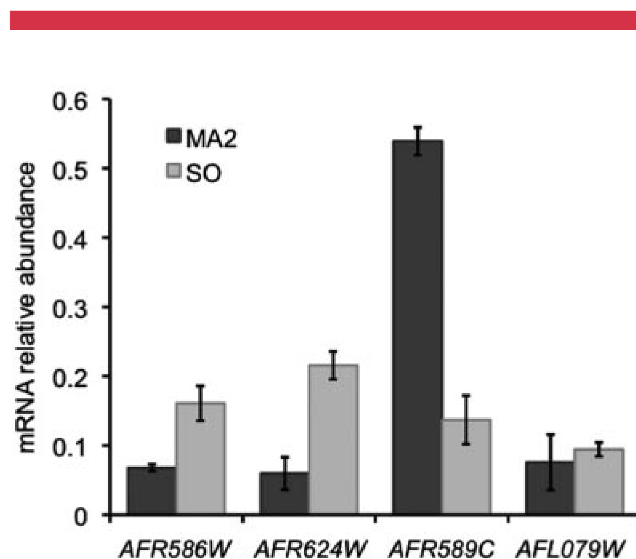

Figure 3. Transcription levels of predicted elongases and desaturases from *A. gossypii*. Relative transcription levels of the *AFR586W*, *AFR624W*, *AFR589C*, and *AFL079W* genes in the wild-type strain of *A. gossypii* grown in two different media (MA2 and SO). Transcription levels were normalized using the *A. gossypii ACT1* gene as a reference. Relative quantitative analyses were performed using LightCycler^{TM} 480 software. The results are means of two independent experiments performed in duplicate and are expressed as a ratio of the cDNA abundance of the target genes with respect to the *ACT1* mRNA levels.

fourfold reduction) when soybean oil was used as the sole carbon source (Fig. 3). This might be explained in terms of the highly unsaturated FA content of soybean oil, which contains 55% of linoleic acid, the product of Δ12 desaturases. Feedback regulation by end-products has been described previously for other desaturases such as *OLE1* in *S. cerevisiae* (Martin et al., 2007). No significant differences between the two culture conditions were found in the transcriptional rate for *AFL079W*.

Tuning FA Composition in *A. gossypii*

The FA profile of an organism depends on both the desaturation and elongation systems. Accordingly, the modification of these enzymatic systems results in a tuning of the FA profile. We have recently described that the FA profile of *A. gossypii* grown in glucose as the sole carbon source is characterized by 80% of unsaturated FAs (Ledesma-Amaro et al., 2014). Oleic acid (55%), palmitoleic acid (20%), and palmitic acid (14%) are the most abundant FAs in *A. gossypii*, followed by linoleic acid (3%), stearic acid (1,1%), and around 4% of very long-chain fatty acids. With a view to broadening the industrial application of the *A. gossypii* SCOs, it was decided to carry out genetic engineering modifications of the elongation and desaturation systems of *A. gossypii* by both gene deletion and overexpression of the *AFR586W*, *AFR624W*, *AFR589C*, and *AFL079W* genes, hereafter referred to as *AgELO586*, *AgELO624*, *AgDES589*, and *AgDES079*, respectively.

All the gene knock-outs were carried out by gene-replacement of the corresponding ORFs with a G418^R selection marker. The gene overexpressions were performed by homologous recombination of an integrative cassette harboring a selection marker and the strong promoter p*AgGPD*. The genomic integration of both the deletion and overexpression cassettes was confirmed by analytical PCR (data not shown). Gene overexpression was verified by qRT-PCR analysis of each strain (data not shown). Following this, the total FA content of the four knock-out strains (*elo586*△, *elo624*△, *des589*△, and *des079*△), the four overexpressing strains (*GPD-ELO586*, *GPD-ELO624*, *GPD-DES589*, and *GPD-DES079*) and the wild-type strain was examined by GC-MS analysis (Table I).

In good agreement with our previous results, strain *elo624*△ showed a strong decrease in very long-chain FAs and, consequently, a significant accumulation of oleic acid (18:1), which was 17% higher than that observed in the wild-type (Fig. 4A and Table I). Only traces of very long-chain FAs were found in the *elo624*△ mutant, thus confirming an FA elongase activity that specifically elongates FAs from 18C to 20–22C. Conversely, the *elo586*△ mutant was totally devoid of very long-chain 24:0 and 26:0 FAs, but a significant accumulation of 20C and 22C FAs (3% of the total FA content) was detected (Fig. 4A and Table I). Accordingly, it may be concluded that AgELO586 catalyzes the elongation of 22:0 and 24:0 FAs with high efficiency; however, the presence of traces of very long-chain FAs in the *elo624*△ mutant indicates that AgELO586 is able to elongate 18:0 FAs with very low efficiency.

The analysis of the *des589*△ strain revealed the absence of linoleic acid (18:2), which is consistent with the lack of △12 desaturase activity (Fig. 4A and Table I). Additionally, no significant changes were found in the FA profile of the *des079*△ strain (data not shown).

Overexpression of the *AgElO624* gene induced an increase in some very long-chain FAs such as C20:1, C22s, and C24s (Fig. 4B and Table I) and, as a result, a decrease in the level of C18s. In contrast, strain *GPD-ELO586* showed a fourfold increase in the proportion of cerotic acid (26:0) and a general decrease in FAs shorter than C26 (Fig. 4B and Table I). Also, the overexpression of *AgDES589* triggered a strong increase in linoleic acid levels (18:2; fivefold higher than the wild-type)

Table I. Molecular percentage of fatty acids in the *A. gossypii* strains.

Fatty acid	WT	GPD-ELO624	GPD-ELO586	GPD-DES589	elo624Δ	elo586Δ	des589Δ
12:0	—	0.17 ± 0.14	—	—	—	—	—
14:0	0.24 ± 0.04	1.13 ± 0.06	0.47 ± 0.28	0.45 ± 0.20	0.11 ± 0.04	0.46 ± 0.01	0.48 ± 0.01
14:1	—	0.26 ± 0.24	—	—	—	—	—
15:1	—	0.03 ± 0.02	—	—	—	—	—
15:0	—	0.17 ± 0.07	—	—	—	—	—
16:0	14.39 ± 1.35	14.71 ± 0.15	16.14 ± 0.60	15.50 ± 0.94	14.32 ± 0.17	17.48 ± 0.27	17.30 ± 1.18
16:1	20.85 ± 0.74	28.30 ± 0.05	22.39 ± 0.88	24.21 ± 0.13	13.96 ± 0.10	23.09 ± 0.35	22.12 ± 1.07
17:0	0.24 ± 0.16	0.07 ± 0.1	0.07 ± 0.11	—	0.23 ± 0.06	0.17 ± 0.06	0.16 ± 0.01
17:1	1.54 ± 0.18	1.04 ± 0.03	1.84 ± 0.09	0.62 ± 0.05	1.22 ± 0.06	0.72 ± 0.01	1.20 ± 0.18
18:0	1.11 ± 0.17	0.59 ± 0.06	0.65 ± 0.06	1.72 ± 0.52	1.38 ± 0.03	0.71 ± 0.08	2.14 ± 0.04
18:1	55.10 ± 0.55	45.45 ± 0.57	51.84 ± 0.75	35.37 ± 1.99	64.83 ± 0.69	53.26 ± 0.61	52.08 ± 0.75
18:2	2.97 ± 0.98	0.59 ± 0.14	1.95 ± 0.17	15.91 ± 3.1	3.56 ± 0.17	1.16 ± 0.03	—
20:0	0.01 ± 0.01	0.01 ± 0.01	—	0.08 ± 0.07	—	—	0.17 ± 0.01
20:1	0.62 ± 0.12	1.83 ± 0.08	—	1.77 ± 0.15	—	1.49 ± 0.06	0.62 ± 0.14
22:0	0.16 ± 0.13	0.31 ± 0.02	—	0.11 ± 0.01	—	1.47 ± 0.51	0.11 ± 0.05
22:1	—	0.20 ± 0.02	—	—	—	—	—
24:0	1.72 ± 0.39	3.01 ± 0.36	0.13 ± 0.19	2.09 ± 0.29	0.28 ± 0.11	—	2.28 ± 0.41
24:1	—	1.45 ± 0.08	—	0.22 ± 0.08	—	—	0.19 ± 0.12
26:0	1.06 ± 0.13	0.69 ± 0.11	4.50 ± 1.14	1.95 ± 0.02	0.02 ± 0.01	—	1.16 ± 0.24

All the data are expressed as means of three experiments ± standard deviation.

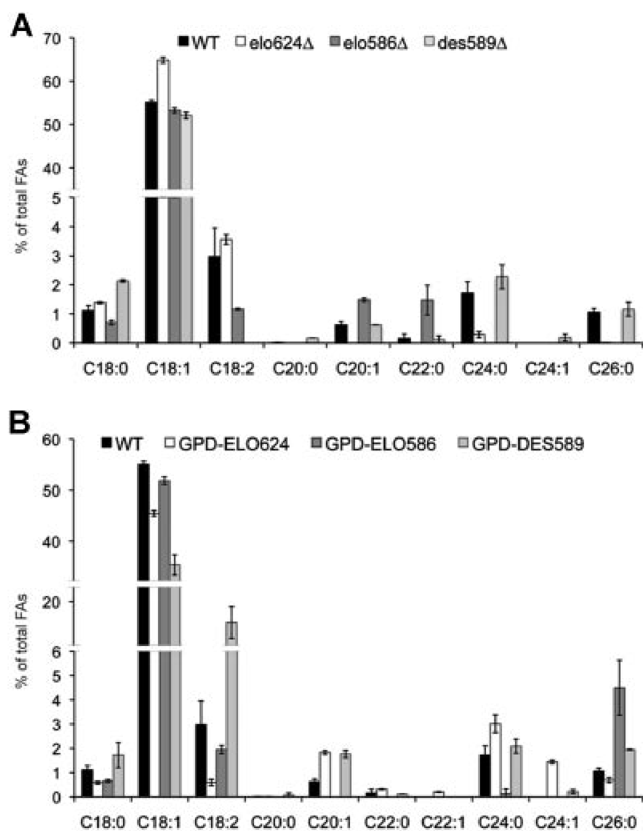

Figure 4. Fatty acid profile in the engineered *A. gossypii* strains. **A**: Relative abundance of long- and very long-chain FAs in strains *elo586*△, *elo624*△, and *des589*△. **B**: Relative abundance of long- and very long-chain FAs in strains *GPD-ELO586*, *GPD-ELO624*, and *GPD-DES589*. The FA profile of the wild-type strain was used for comparison. The results are means of two independent experiments performed in duplicate. Error bars represent standard deviations.

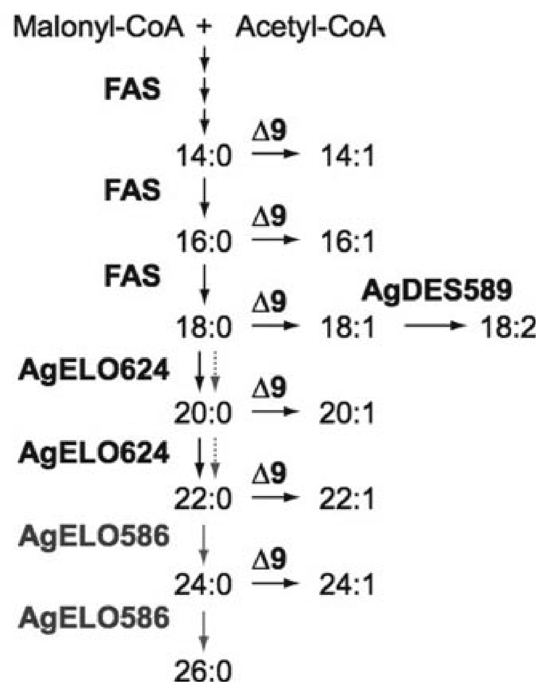

Figure 5. Proposed model of the FA metabolism in *A. gossypii*. The gray arrows indicate reactions that can be catalyzed by AgELO586. Dashed arrows denote that AgELO586 can elongate both 18:0 and 20:0 with low efficiency. FAS, FA synthase; △9, first desaturase △9.

(Fig. 4B and Table I). These levels of linoleic acid (about 16% of the total FA content) are much higher than the levels of linoleic acid that resulted after the heterologous expression of *AgDES589* in *S. cerevisiae* (see Table SII). Hence, *S. cerevisiae*, which naturally lacks PUFAs, may not be the best model for the study of FA desaturases. Again, strain GPD-DES079 did not show any significant change in the FA profile (data not shown). Taken together, our results demonstrate that *A. gossypii* is endowed with two putative elongases, namely *AgELO624* and *AgELO586* that are able to elongate a C18:0 FA up to C22:0 and C:26:0, respectively. Moreover, the *A. gossypii* desaturation system comprises a △12 desaturase encoded by the gene *AgDES589* and also a △9 desaturase activity. The activity encoded by the gene *AgDES079* remains elusive. Our results indicate that it is unlikely that *AgDES079* would code for a △6 FA desaturase; in contrast, sequence similarity pointed to the possibility that AgDES079 might be involved in the △8 desaturation of sphingolipids. Based on our evidence, a complete map of FA elongation and desaturation system for *A. gossypii* can be proposed (Fig. 5). In our model, FAs would use malonyl-CoA and acetyl-CoA to form and elongate a saturated FA of up to 18 carbons in length. Saturated FAs would be desaturated by a Δ9 desaturase. The C18:0 FA could be the substrate for three enzymes: two elongases (AgELO624 and AgELO586) and the Δ9 desaturase. The C18:0 FA would be mainly transformed into C18:1 (oleic acid), which accounts for more than 50% of the total FA content. Oleic acid is the substrate of the △12 desaturase (AgDES589), which can form linoleic acid (18:2). The remaining oleic acid would be elongated either by AgELO624 up to C20-22 or by AgELO586, which can elongate FAs up to 24:0 or 26:0.

Applications of Engineered Strains of *A. gossypii* With Modified FA Profiles

Metabolic engineering of the elongation and desaturation systems of *A. gossypii* uncovered significant differences in the SCO profiles of the engineered strains. These might well have different applications in biotechnology.

Strain *GPD-DES589* was able to accumulate high amounts of linoleic acid (18:2), an essential fatty acid for humans, which is a precursor of both omega-3 and omega-6 FAs. Strain *GPD-ELO586* was characterized by the accumulation of cerotic acid (26:0), which is the main component of high-melting point waxes such as carnauba wax, widely used in polishes, cosmetics, the food industry, paper coating and drug delivery (Budai et al., 2012; Hoashi et al., 2012). In addition, the strain

overexpressing the elongase AgELO624 contained the uncommon nervonic acid (24:1), which is a major component of high-temperature lubricants and engineered nylons, and it is also important for pharmaceuticals and nutraceuticals (Taylor et al., 2009).

SCOs can be transesterified directly to produce fatty acid methyl esters (FAMEs), which are good candidates for biodiesel production (Caspeta and Nielsen, 2013). The properties of biodiesel mostly depend on the degree of oil refinement (pre-treatment), the transesterification process, and the quality of the purification steps. However, certain other critical parameters of biodiesel also rely on the FA composition and hence a critical range of FAs in the SCO can be considered for optimal biodiesel efficiency (Ramos et al., 2009). Thus, neither high levels of polyunsaturated FAs nor long-chain saturated FAs are desirable for biodiesel, for two reasons (i) unsaturations are highly unstable and their presence negatively affects some important biodiesel parameters such as the iodine value and the cetane number; and (ii) long-chain saturated FAs increase the cold filter plugging point, a parameter that is related to the low-temperature flow properties of biodiesel. Consequently, the SCOs used for biodiesel production must have high oxidative stability and a low cold-flow value (Wu et al., 2012). Incidentally, some of our engineered strains might have improved biodiesel properties, especially with regard to the reduction of both PUFAs and long-chain saturated FAs. Accordingly, we decided to further analyze the theoretical application of the FAMEs from our *A. gossypii* mutant strains for the production of biodiesel.

Several biodiesel quality standards have been established in different countries, including the USA (ASTM D 6751) and Europe (EN 14214 [vehicle use] and EN14213 [heating oil]). Here the theoretical properties of the *A. gossypii* SCO for biodiesel production were analyzed according to the most restrictive standard, EN 14214. As stated above, the quality of biodiesel depends on the FA composition of the oil (Ramos et al., 2009). We therefore evaluated the following parameters of the *A. gossypii* SCOs (Table SIII): (a) kinematic viscosity at 40°C, (b) density at 15°C, (c) iodine value, (d) heating value, (e) cetane number, and (f) cold filter plugging point. The wild-type strain of *A. gossypii* already satisfied the standards for all the parameters with the exception of the cold filter plugging point.

The cold filter plugging point (CFPP) indicates the lowest temperature for biodiesel to flow through the filters and fuel systems. The CFPP of biodiesel mostly depends on both the content of saturated esters and the length of the acyl chain (Wu et al., 2012). The EN 14214 standard does not specify values for optimal CFPP; this is established by national regulations according to the climatic conditions of each country (Knothe, 2006). Most of the *A. gossypii* SCOs evaluated displayed undesirable CFPP values (between 9.7 and 25.8) due to the high amount of VLCFAs; however, both strain *elo586*△ (−2.9) and, especially, strain *elo624*△ (−7.9) showed optimal CFPP values, making them promising candidates for biodiesel SCOs (Table SIII).

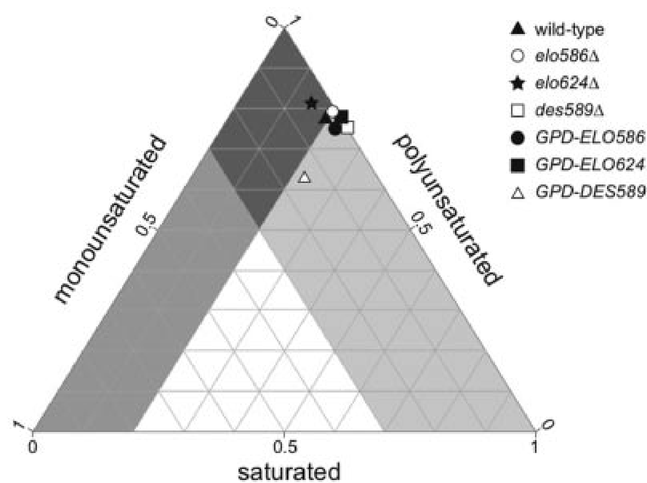

Figure 6. Triangular graph of biodiesel from *A. gossypii* strains. Monounsaturated, polyunsaturated, and saturated methyl ester biodiesels from *A. gossypii*. Each vertex of the triangle corresponds to 100% of monounsaturated, polyunsaturated and saturated methyl ester composition, respectively. Areas satisfying the EN14214 standard: light gray (right), good cetane number, and iodine value; mid-gray (left), good CFPP; and dark gray (intersection), biodiesel with theoretically optimal properties that satisfied the EN14214 standard.

We also plotted the *A. gossypii* SCOs according to their composition in monounsaturated, polyunsaturated, and saturated methyl esters on a triangular graph for additional prediction of their biodiesel properties, as described elsewhere (Ramos et al., 2009) (Fig. 6). We found that most of the FAMEs from our *A. gossypii* strains fell within the borderline of the area that correlates with good biodiesel properties. However, strain *elo624*△ remained close to the center of the optimal area, again suggesting that the biodiesel from the SCO of the *elo624*△ strain might be the most suitable for use as a biofuel. As discussed above, the reduction in VLCFAs in the *elo624*△ strain that would determine an optimal CFPP value, as well as the good properties regarding oxidative stability, suggest that this strain could be a potential tool for biodiesel production.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Artículo 7:

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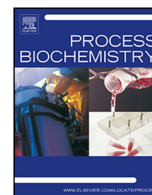

Review

Biotechnological production of feed nucleotides by microbial strain improvement

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ABSTRACT

Sodium salts of inosine monophosphate (IMP) and guanosine monophosphate (GMP) are potent flavour enhancers. They are widely used as food additives in combination with monosodium glutamate (MSG) to synergistically increase *umami* flavour. In recent years, both inosine and guanosine derivatives have gained further importance because of their beneficial effects, related to their antioxidant, neuroprotective, cardiotonic and immunomodulatory properties. The industrial production of both IMP and GMP is mainly achieved either by RNA breakdown and nucleotide extraction or by microbial fermentation using different microorganisms such as *Corynebacterium*, *Bacillus*, or *Escherichia coli*. This work reviews the metabolic pathways and regulatory networks of purine synthesis, including both IMP and GMP, and the biotechnological processes applied to the production of these compounds, ranging from classical random mutagenesis to rational design by metabolic engineering. Recent advances of systems biology approaches, along with the rapid development of synthetic biology, may offer a basis for future manipulations to further increase the productivity of the fermentation processes.

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1. Introduction

In cellular physiology inosine monophosphate (IMP) and guanosine monophosphate (GMP) are two essential metabolites. In addition, these purine nucleotides are of significant economical interest for the applied biotechnology industry because they are currently used as foodstuff additives.

Abbreviations: AMP, adenosine monophosphate; CMP, cytidine monophosphate; GMP, guanosine monophosphate; GDP, guanosine diphosptate; GTP, guanosine triphosphate; ppGpp, guanosine tetraphosphate; cGMP, cyclic GMP; IMP, inosine monophosphate; UMP, uridine monophosphate; XMP, xanthosine monophosphate; sAMP, adenylosuccinate; AIR, aminoimidazole ribotide; MSG, monosodium glutamate; CNS, central nervous system; GRAS, generally recognized as safe; PRPP, phosphoribosyl pyrophosphate.

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1.1. Physiological relevance of IMP and GMP

Nucleotides and nucleosides are essential elements of life since they are the structural components of both DNA and RNA, energy carriers (*i.e.* ATP and GTP), enzyme cofactors (*i.e.* NAD^+ and $NADP^+$) and some vitamins (vitamin B1, B2 and B9). Therefore, nutritional deficiencies of purines and alterations in the nucleotide metabolism may cause different severe diseases in humans [1,2].

IMP is the central molecule of purine biosynthesis, and hence its biosynthesis is tightly regulated at different levels. GMP is synthesized either from IMP through the *de novo* purine pathway or from guanine through the *salvage* purine pathways, and it is the precursor molecule of GDP, GTP, ppGpp and cGMP, whose biological importance is well known [3,4].

1.2. Industrial relevance of IMP and GMP

GMP, IMP, guanosine and inosine have sparked interest in applied biotechnology and the food industry because they are widely used as flavouring additives in food technology and the pharmaceutical industry.

The market for flavouring molecules has undergone significant advances during the past few years and is now one of the most promising areas of research in food technology. Monosodium glutamate (MSG) is the flavour additive most studied and is currently used to potentiate *umami* taste in foods by providing meaty and savoury flavours [5]. IMP and GMP are also naturally occurring sources of *umami* taste that are often used in combination with glutamate salts to enhance *umami* flavour synergistically [6].

The worldwide consumption of flavouring purine derivatives underwent a significant increase from 4465 tm in 1992 to over 9000 tm in 1999 [7]. Consequently, the total worldwide nucleotide production capacity increased from 6600 tm in 1992 to approximately 10,700 tm in 1997 [7] and hence the world nucleotide market reached 15,000 tm in 2005 and was estimated to be 22,000 tm in 2010 (http://www.foodnavigator.com/Financial-Industry/Ajinomoto-ramps-up-MSG-production-on-market-growth). Currently, there are four main industrial producers of nucleotides: Ajinomoto (Japan), Takeda (Japan), Cheil (South Korea) and Daesang (South Korea).

1.3. Health and nutritional aspects

Besides the intrinsic flavouring activity of guanylate and inosinate derivatives, some nutritional benefits associated with their use as food additives have been reported. Umami taste additives such as IMP and GMP can serve either to increase the palatability of some flavourless foods or to improve the acceptance of low-salt diets. In this regard, the loss of taste and smell in elderly people, which can contribute to certain nutritional deficits, can be attenuated by the addition of flavour enhancers in their diets, thus favouring the intake of proteins and essential vitamins and minerals [5,8].

According to the U.S. FDA, flavour enhancers are substances designated as GRAS (Generally Recognized As Safe), but they have also been reported to have certain beneficial effects for human health. For example, inosine has been reported to stimulate axon growth in the adult central nervous system [9]. It has also been described as being neuroprotective, cardiotonic and immunomodulatory [10–12]. Besides, extracellular guanosine can exert neurotrophic and neuritogenic effects, which are important for neuronal development [13]. In addition, some nucleoside derivatives are potent antivirals and they have been proposed as chemotherapeutic agents [14]. Finally, both guanosine and inosine have been reported to display antioxidant activities, which can serve to protect cells against reactive oxygen species [15].

Here we review the metabolic pathways for the production of both IMP and GMP. We also recapitulate the biotechnological processes and the producing microorganisms that are currently employed in the industrial elaboration of these purine nucleotides.

2. Purine biosynthesis: general pathway

Both IMP and GMP are metabolic products of the purine biosynthetic pathway. The biosynthesis of purines can be achieved through *de novo* synthesis from PRPP and glutamine. Alternatively, purine nucleobases can be transformed directly to their nucleoside monophosphate derivatives through the *salvage* pathways (Fig. 1), which also involve the consumption of PRPP [3,4,16]. Hence, the synthesis of PRPP from ribose-5-phosphate channels metabolic flux towards the biosynthesis of purines [17,18].

The *de novo* purine pathway (Fig. 1) leads to the conversion of PRPP and glutamine into IMP through 10 different enzymatic reactions. Thus, IMP is the central metabolite of the purine pathway and it can be subsequently converted either to AMP or GMP in two successive metabolic steps catalyzed by enzymes specific to each nucleotide [3,4]. The AMP branch consists of two enzymatic reactions controlled by sAMP synthetase and sAMP lyase, which convert the IMP into sAMP, and sAMP into AMP, respectively. In the other branch of the purine pathway, IMP dehydrogenase converts IMP into XMP, and GMP synthase transforms XMP into GMP (Fig. 1) [3,4].

The intracellular pool of PRPP can also be used for the recycling of nucleobases through the purine *salvage* pathways, which in some parasitic organisms is the only pathway to purine nucleotides. In the *salvage* pathways, the PRPP is transferred into free nucleobases by specific phosphoribosyltransferases (Fig. 1). The enzymatic activities that have been described in the *salvage* pathways are as follows: adenine phosphoribosyltransferase, hypoxanthine phosphoribosyltransferase, xanthine phosphoribosyltransferase and guanine phosphoribosyltransferase, which transform adenine, hypoxanthine, xanthine and guanine into AMP, IMP, XMP and GMP, respectively [3,19].

Furthermore, some nucleobases and nucleotides can be interconverted by specific oxidases and deaminases (Fig. 1). Adenine deaminase transforms adenine into hypoxanthine [20]; guanine deaminase converts guanine into xanthine [21]; and xanthine dehydrogenase/oxidase catalyzes the bidirectional conversion between xanthine and hypoxanthine [22]. Other alternative routes of interconversion among virtually all purine derivatives have also been described in several organisms (Fig. 1). Examples of such enzymatic activities are AMP deaminase, which converts AMP into IMP [23]; GMP reductase, which transforms GMP into IMP [24]; AMP nucleosidase, which hydrolyzes AMP to form ribose-5-phosphate and adenine [25]; adenosine deaminase, which transforms adenosine into inosine [26]; 5′-nucleotidase, which converts GMP, XMP, IMP and AMP into guanosine, xanthine, inosine and adenosine, respectively [27,28]; inosine/guanosine kinase and adenosine kinase, which catalyze the inverse reaction for the conversion of nucleosides into nucleotides [29,30] and finally, purine nucleoside phosphorylase (PNP), which metabolizes nucleosides into their nucleobases [31].

The purine biosynthetic network is connected to other important metabolic pathways, such as those involved in the biosynthesis of riboflavin, folic acid, thiamine, glycine, serine, threonine, histidine, alanine, aspartic acid, glutamic acid, glyoxylate and dicarboxylate [32–34]. Indeed, the overproduction of vitamins such as riboflavin and folic acid has been achieved by metabolic engineering of the purine pathway in order to increase metabolic flux towards the biosynthesis of the immediate precursors of the vitamins [16,18,35].

Fig. 1. Purine biosynthesis: the general pathway. PRPP, Phosphoribosyl pyrophosphate; IMP, inosine monophosphate; XMP, xanthosine monophosphate; GMP, guanosine monophosphate; sAMP, adenylosuccinate; AMP, adenosine monophosphate. Numbers indicate enzymatic activities: 1, Ribose-phosphate pyrophosphokinase (PRPP synthetase); 2, Series of ten reactions towards IMP synthesis; 3, Adenylosuccinate synthase; 4, Adenylosuccinate lyase; 5, IMP dehydrogenase; 6, GMP synthase; 7, Guanine phosphoribosyltransferase; 8, Xanthine phosphorybosiltransferase; 9, Hypoxanthine phosphoribosyltransferase; 10, Adenine phosphoribosyltransferase; 11, Guanine deaminase; 12, Xanthine dehydrogenase/oxidase; 13, Adenine deaminase; 14, AMP deaminase; 15, GMP reductase; 16, AMP nucleosidase; 17, Adenosine deaminase; 18, 5′-nucleotidase; 19, Adenosine kinase; 20, Inosine/guanosine kinase; 21, Purine-nucleoside phosphorylase; 22, Transketolase. Black arrows indicate an enzymatic process; dashed arrows indicate a multi-step pathway, and grey arrows indicate other metabolic connections outside the pathway. Bold arrows and numbers indicate metabolic engineering described in the text.

3. Purine biosynthesis in nucleotide-overproducing organisms

In most organisms, central metabolic flux in the purine biosynthetic pathway is very similar; however, some differences with regard to the above general purine pathway can be found among microorganisms that are widely used in the biotechnology industry, such as *B. subtilis*, *E. coli*, *S. cerevisiae* and *C. glutamicum*.

In eukaryotes, the sixth reaction of the *de novo* purine pathway, *i.e.* the transformation of AIR (aminoimidazole ribotide) into CAIR (Carboxyaminoimidazole ribonucleotide), is catalyzed by a bifunctional enzyme that is the product of a fused gene (*ADE2* in *S. cerevisiae*), which in most bacterial genomes is organized as two separate ORFs (*purK* and *purE* in *E. coli*) [36]. There is no evidence of AMP deaminase activity in bacteria, and hence the conversion of AMP into IMP would not be possible [23].

The direct transformation of GMP into AMP is achieved by GMP reductases, which are found in some organisms such as *E. coli*, *B. subtilis*, *Salmonella typhimurium* and humans [37]. However, this enzyme is absent in *S. cerevisiae* and *Corynebacterium* sp.

Some of the enzymes that catalyze interconversion among purine nucleobases are lacking in some organisms. For example, most species of the genus *Corynebacterium* completely lack the enzymes for nucleobase conversion. Also, xanthine dehydrogenase/oxidase, which has been described in humans and other metazoan genomes [22], is absent in *S. cerevisiae* and *Corynebacterium* sp. In contrast, *E. coli* and *B. subtilis* only lack xanthine oxidase activity and therefore xanthine cannot be transformed into hypoxanthine in these microorganisms.

AMP nucleosidase activity is only found in prokaryotes [25], although *B. subtilis* lacks this enzyme.

Adenosine deaminase is encoded in mammalian genomes and it is also present in some bacteria such as *Streptomyces coelicolor* and *E. coli* [26,38]. However, it is absent in *B. subtilis* and *Corynebacterium* sp., and its presence remains elusive in the *S. cerevisiae* genome [39].

The 5′-nucleotidases that catalyze the hydrolysis of nucleotides into nucleosides display significant differences in both substrate specificity and cellular location among different species. For example, this enzyme is secreted in some organisms such as *C. glutamicum* and *B. subtilis* [40,41]. In contrast, the genome of *S. cerevisiae* encodes a cytosolic 5′-nucleotidase, which is specific for the conversion of IMP into inosine [28].

In most prokaryotic organisms, the conversion of nucleosides into nucleotides is catalyzed by an inosine/guanosine kinase that transforms both inosine and guanosine into IMP and GMP, respectively [30]. Also, the adenosine kinase activity that transforms adenosine into AMP in eukaryotic organisms has been reported [29,42]. Finally, purine nucleoside phosphorylase enzymes that recycle free nucleobases from nucleosides are present in most organisms [31,43].

4. Regulation of purine biosynthesis

Owing to the pivotal roles of purines in cell physiology, the pool of intracellular purine nucleotides must be maintained under strict control and hence the biosynthesis of purines is a tightly regulated pathway for cellular homeostasis and energy supplies to be maintained. In eukaryotes, most of the genes for both AMP and GMP synthesis are negatively regulated at transcriptional level by extracellular purines [44]. A metabolic level of regulation also exists in some steps of the purine pathway by end product-feedback regulation of enzymatic activities. In particular, PRPP amidotransferase is inhibited by monophosphate nucleotides (AMP, GMP and IMP); adenylsuccinate synthetase is inhibited by AMP; and IMP dehydrogenase is inhibited by GMP [45–47].

PRPP amidotransferase is the rate-limiting enzyme in the *de novo* purine pathway and it is regulated at both the transcriptional and metabolic level [16,48]. Also, the transcription of the *ADE* genes that are involved in AMP biosynthesis are repressed by extracellular adenine and are activated by the transcription factors Bas1 and Bas2 (Pho2) [19]. While the synthesis of both AMP and IMP is co-regulated at the transcriptional level, the genes for GMP synthesis are specifically regulated by a guanine response element (GRE), which is repressed either by guanine, GDP or GDP derivatives [44].

In some organisms such as *E. coli* and *B. subtilis* most of the genes involved in the purine pathway are organized in operons (*pur* operon) [49]. The *purR* gene is the main regulator of both the *pur*

operon and other genes related to purine biosynthesis. The activity of PurR can be enhanced to repress the expression of target genes when purine nucleotides become available from the environment [50,51]. The effectors acting on *purR* expression vary among species, and some functional differences can also be found in the role of the PurR repressor in *E. coli* and *B. subtilis* [48]. Additionally, it has been reported that the expression of the *pur* operon of *B. subtilis* is further controlled by a guanine-sensing riboswitch that negatively regulates the transcription of the structural genes of the operon [52].

5. Producing organisms

All living organisms are able to synthesize purines, although some parasites lack a *de novo* biosynthesis pathway and their purine supply relies exclusively on the *salvage* pathways (see below) [53]. The yield of purine derivatives is very heterogeneous among different species. Accordingly, it is necessary to define a "purine nucleotide-producing organism" not only in terms of its ability to synthesize purine derivatives, but, more importantly, in terms of its capacity to accumulate purines for competitive industrial advantages. This include several biotechnological aspects such as the production of a high yield of the desired compound, the preferential accumulation of the final molecules (IMP or GMP) instead of their intermediates (inosine or guanosine), the ability to excrete the desired compound into the culture medium, and/or the intrinsic industrially-friendly properties of the organism.

The high productivity of the so called "purine nucleotide-producing organisms" can be enhanced considerably either by random mutagenesis or by metabolic engineering. Consequently, it is crucial to have knowledge of the genetics and regulatory mechanisms of the purine pathway, as well as the genome sequence, together with adequate molecular tools to implement genetic modifications in those organisms.

B. subtilis, *C. glutamicum* and *C. ammoniagens* have traditionally been regarded as the microorganisms with the highest potential for ribonucleotide production [54]. These microorganisms are able to accumulate large amounts of nucleosides in the culture medium, thus avoiding complex and expensive recovery steps. Other microorganisms have also been used for the production of GMP and IMP. For example, GMP has been produced in Streptomycetes species [55] and the production of IMP has been carried out in *Micrococcus sodonensis* and *Arthrobacter citreus* [56]. More recently, *Bacillus amyloliquefaciens* has also been proposed for the production of nucleosides with yields that may be comparable to those of *B. subtilis* [57]. *E. coli* is also considered as a potential purine nucleotide-producing organism owing to the excellent availability of molecular biology and genomic tools for this model organism, which allow the engineering of *E. coli* strains for the overproduction of purine nucleotides [58].

To date, eukaryotic microorganisms such as *Candida*, *Pichia*, *Saccharomyces* or *Kluiveromyces* have only been considered for the industrial production of purine nucleotides from RNA but not from direct fermentation. Indeed, *S. cerevisiae*, which is the paradigm of unicellular eukaryotic model organisms, is far from being in a situation in which it would be able to produce enough inosine and guanosine nucleotides to meet the industrial requirements for a biotechnological process.

6. Industrial production and strain engineering

The industrial production of IMP and GMP has been carried out for their use as flavour enhancers [59] and to date several methods have been developed to improve the production process. There are two main approaches used for the production of IMP and GMP: 1) RNA breakdown and nucleotide extraction; and 2) the microbial production of nucleotides and nucleosides by fermentation.

6.1. Nucleotide production by RNA breakdown

The first method is based on RNA extraction from cell cultures and subsequent hydrolysis to obtain free nucleotides. Cells can either be cultivated for this aim or can be obtained as by-products from other fermentation processes [60].

Most yeasts used for RNA production are those with a high RNA content, such as *Candida*, *Pichia*, *Saccharomyces* or *Hansenula*, which have been studied and modified for the overproduction of nucleotides [61,62]. Autolysis of yeast cultures is induced by heat treatment and RNA isolation is carried out by ethanol precipitation. Subsequently, the extraction of free nucleotides is achieved by either chemical or enzymatic hydrolysis. For the chemical hydrolysis of RNA, samples are treated with $Ca(OH)_2$ to induce nucleic acid disruption. In contrast, enzymatic methods require either soluble or immobilized 5′-phosphodiesterase activity to obtain free nucleotides from RNA [63]. The most widely used enzyme for this purpose is the Nuclease P1 from *Penicillium* [64].

The main drawback of the production of IMP and GMP from RNA is the relatively high abundance of undesirable by-products such as AMP, CMP and UMP, which lack any flavouring activity. Consequently, enzymatic deamination of AMP to IMP is required by adenyl deaminase activity [65] as well as removal of both CMP and UMP by activated carbon adsorption [66].

6.2. Strain improvement: mutagenesis

The second method consists of the direct production of nucleotides and nucleosides from cell cultures by microbial fermentation of improved strains obtained by several rounds of random mutagenesis (summarized in Table 1).

Inosine and guanosine are usually produced and recovered by fermentation prior to their phosphorylation to form their monophosphate derivatives by chemical or enzymatic treatments [67]. Exceptionally, several mutagenesis-based approaches have been developed to produce IMP, XMP and GMP directly [54]. IMP overproduction has been described after the mutagenesis of adenine auxotrophic mutants of both *B. subtilis* and *C. ammoniagenes* with either low 5′-nucleotidase activity or increased plasma membrane permeability, an IMP yield between 20 and 27 g/L. Likewise, XMP has also been overproduced in adenine and guanine auxotrophic mutants with weak 5′-nucleotidase activity in different species such as *C. glutamicum*, *C. ammoniagenes* and *B. subtilis*, with maximal production rates of 19 g/L of XMP. In contrast, the direct production of GMP has not yet been reported and, consequently, GMP is obtained from XMP-overproducing strains following a two-step protocol in which XMP is enzymatically converted into GMP by an XMP aminase. The most effective producer strain yields 34.8 mg/ml from 40 mg/ml XMP [54].

Overproduction of nucleosides has also been described after random mutagenesis in either guanine or adenine auxotrophic backgrounds of *B. subtilis* and *C. ammoniagenes* strains. Inosine overproduction has been reported in adenine auxotrophs of *B. subtilis* (20.6 g/L) with both increased 5′-nucleotidase and low IMP dehydrogenase enzymatic activities. Also, an adenine-guanine double auxotrophic mutant of *B. subtilis* is able to produce 17.5 g/L of xanthosine. In addition, a production of 38.7 g/L of inosine has been reported in *C. ammoniagenes* guanine auxotrophic mutant strains that also lack purine nucleoside-degrading activities [54].

The accumulation of high levels of guanosine has been observed in mutants of *B. subtilis* with either high levels of IMP dehydrogenase and low GMP reductase activities or lacking purine nucleoside phosphorylase activities. In this regard, an increased guanosine

Table 1
Random mutagenesis for nucleotide/nucleoside accumulation.

Organism	Strain	Modification	Main product(s)	Ref.
B. subtilis	Adenine auxotroph	Low 5′-nucleotidase activity	IMP	[54]
	Adenine auxotroph	High PM permeability	IMP	[54]
	Adenine, guanine auxotrophs	Low 5′-nucleotidase activity	XMP	[54]
	Adenine auxotroph	High 5′-nucleotidase; low IMP dehydrogenase	Inosine	[54]
	Adenine, guanine auxotrophs	Not described	Xanthosine	[54]
	Adenine auxotroph	High IMP dehydrogenase; low GMP reductase; lacking PNP	Guanosine	[54]
C. ammoniagenes	Adenine auxotroph	Low 5′-nucleotidase activity	IMP	[54]
	Adenine auxotroph	High PM permeability	IMP	[54]
	Adenine, guanine auxotrophs	Low 5′-nucleotidase activity	XMP	[54]
	Guanine auxotrophs	Purine nucleoside -degrading activities	Inosine	[54]
C. glutamicum	Adenine, guanine auxotrophs	Low 5′-nucleotidase activity	XMP	[54]

production (20 g/L) has also been described after abolition of the feedback inhibition of the enzymes IMP dehydrogenase, GMP synthetase, sAMP lyase and PRPP amidotransferase [54].

6.3. *Strain improvement: metabolic engineering*

The genetic and biochemical characterization of the purine pathways in purine nucleotide-producing organisms has afforded a second generation of overproducing strains through genetic engineering means such as gene-targeted mutagenesis and the deregulation of gene expression (see Table 2). These strategies have provided a significant improvement in fermentation processes.

In *E. coli* the overproduction of inosine has been carried out by a combination of gene-deletion and deregulation strategies, leading to the redirection of metabolic flux towards the production of inosine [58]. First, the conversion of inosine was blocked by the inactivation of the *add* (adenosine deaminase), *deoD* (purine nucleoside phosphorylase) and *purA* (adenylosuccinate synthase) genes. Then, the regulation of the purine operon was abolished by gene-disruption of the *purR* regulator and the *purF* gene, which encodes a glutamine PRPP amidotransferase, was replaced by a mutant allele that is not subject to feedback inhibition by its end products. This mutant was able to accumulate up to 1 g/L of inosine from 40 g/L glucose [58]. This strain was also used for the investigation of guanosine accumulation by modifying the *guaB* (inosine 5′ monophosphate dehydrogenase), *gsk* (guanosine-inosine kinase) and *guaC* (guanosine 5′monophosphate reductase) genes. It was seen that *gsk* disruption led to guanosine accumulation in *E. coli*, while mutations in the *guaB* gene did not increase the inosine yield significantly. In contrast, the inactivation of the *guaC* gene decreased inosine concentrations and did not increase guanosine production [68].

The expression of deregulated isoforms of both the PRPP synthetase (*prs*) and PRPP amidotransferase (*purF*) has also been demonstrated to induce a significant increase in the production of inosine in *E. coli* [69]. In addition, the contribution of central carbon metabolism to the production of inosine has been studied in *E. coli* and it has been reported that an increase in the ribose-5-phosphate supply, by disruption of both the *edd* (6-phosphogluconate dehydrase) and *pgi* (glucose-6-phosphate isomerase) genes, also results in the accumulation of inosine. In the same work the authors evaluated the disruption of the *yicP* gene (adenine deaminase), which also leads to inosine accumulation [70,71]. A second purine nucleoside phosphorylase, *xapA*, has also been deleted in *E. coli* to increase inosine production [72]. A combination of the modifications listed above (*purF*$^-$, *purA*$^-$, *deoD*$^-$, *purR*$^-$, *add*$^-$, *edd*$^-$, *pgi*$^-$, *yicP*$^-$, *xapA*$^-$, and mutant *prs*) has been described to afford a maximal inosine production of 7.5 g/L from 40 g/L of glucose in the fermentation medium [69].

In *C. ammoniagenes* the redirection of the carbon flux from the pentose phosphate pathway towards the purine pathway can be achieved by disruption of the *tkt* gene that codes for the transketolase enzyme, and this mutant strain is able to accumulate 10–30% more inosine and XMP than the wild-type strain [73]. *C. glutamicum* has recently been engineered to accumulate IMP by a multiple approach consisting of [74]: (i) increasing the availability of the precursor, ribose 5-phosphate through deletion of the *pgi* gene encoding glucose 6-phosphate isomerase; (ii) de-bottlenecking regulation-restricted branch points by the deregulation of the PRPP amidotransferase *purF* gene, and (iii) deactivating IMP-depleting reactions, such as adenylosuccinate synthetase (*purA*) (catalyzing the conversion of IMP to AMP) and IMP-dehydrogenase (*guaB2*) (catalyzing the reaction of IMP to GMP). The distribution of the intracellular carbon flux in the engineered strains was analyzed by

Table 2
Metabolic engineering for nucleotide/nucleoside accumulation.

Organism	Target gene(s)	Modification	Main product(s)	Ref.
E. coli	*add, deoD, purA, purR, purF*	Deletion, mutant allele (*purF*)	Inosine	[58]
	add, deoD, purA, purR, purF, gsk	Deletion, mutant allele (*purF*)	Inosine, guanosine	[68]
	purF, purA, deoD, purR, add, edd, pgi, yicP, xapA and prs	Deletion, mutant allele (*purF, prs*).	Inosine	[69]
	edd, pgi, yicP	Deletion	Inosine	[70]
	xapA	Deletion	Inosine	[72]
C. ammoniagenes	*tkt*	Deletion	Inosine	[73]
C. glutamicum	*pgi, purA, guaB2, purF*	Deletion, mutant allele (*purF*)	IMP	[74]
B. subtilis	*purA, guaB, punA, deoD, purR, pur operon* (5′-UTR), *pur* operon (promoter)	Deletion, mutant allele (*pur* operon promoter)	Inosine, guanosine	[48]
	purA, deoD,	Deletion	Inosine	[75]
B. amyloliquefaciens	*pbuE, E. coli nepI*	Overexpression	Inosine	[76]

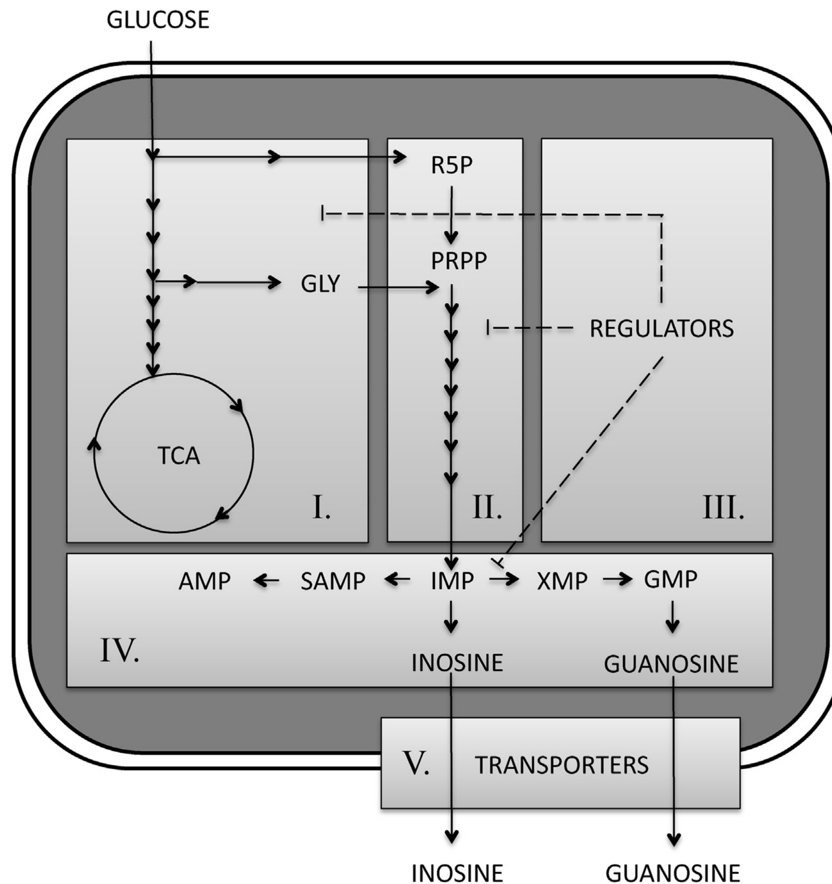

Fig. 2. Clusters of metabolic engineering strategies. I) Central metabolism, II) *de novo* pathway, III) Salvage pathway, IV) Regulator genes, V) Nucleoside/nucleotide transporters. GLY, glycine; R5P, ribose 5 phosphate.

both ^{13}C MFA (Metabolic Flux Analysis) and metabolomic analyses, confirming once again the fine-tuned regulation of the purine pathway. The Δ*purA* strain proved to be the best producer of IMP (44 μmol g_{CDW}^{-1}).

In *B. subtilis* a rational metabolic engineering approach has also been described for the production of inosine and guanosine [48] First, the *purA* (adenylosuccinate synthase) and *guaB* (IMP dehydrogenase) genes were inactivated, thus preventing IMP conversion to either AMP or GMP. Then, the *punA* and *deoD* genes (guanosine/inosine phosphorylases) were also inactivated in order to reduce inosine degradation. Finally, the purine operon was also modified by three additional mutations: the *purR* (purine operon repressor) gene and the 5′-UTR of the operon that contains the guanine riboswitch were disrupted, and the −10 sequence of the *pur* promoter was optimized to increase the transcriptional activity of the purine operon. This engineered strain harbouring the seven mutations described above is able to produce up to 6 g/L of inosine from 30 g/L of glucose in the culture medium [48]. More recently, a metabolic flux analysis of a *B. subtilis deoD purA* double mutant has shown that the inactivation of *deoD* and *purA* contribute additively to the accumulation of inosine [75].

Inosine overproduction has been also demonstrated in *Bacillus amyloliquefaciens* by both the overexpression of the nucleoside transporter *pbuE* and heterologous expression of the homologous gene from *E. coli nepI*, thus offering an interesting modification for future strain engineering [76].

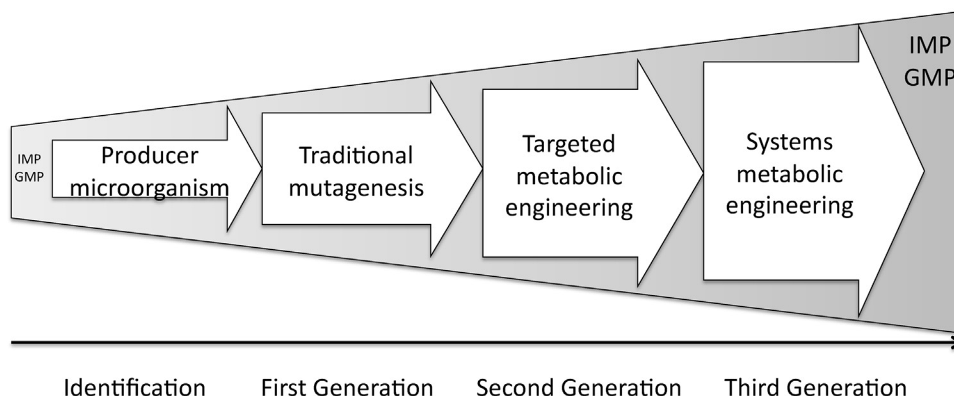

Fig. 3. Time-line evolution of IMP and GMP production by fermentation.

Many patents have been developed to protect strain improvements leading to the accumulation of guanosine or inosine derivatives. The increasing number of patent applications regarding this issue correlates with the importance of this market. Some examples of this patent protection are the use of the genes that code for nucleotide/nucleoside transporters: *yeaS* (*leuE*) of *E. coli* [77] and *ydhL* of *B. subtilis* [78]. Also, inactivation of the gene encoding 3-hexulose-6-phosphate synthase has been reported to improve purine ribonucleoside productivity and this enzyme was patented in 2010 [79].

Production processes have been also protected. An example is the use of a two-step fermentation process, first to produce XMP, guanosine or inosine and, second to produce the nucleotides IMP and GMP [80].

In summary, some common features among the metabolic engineering approaches for inosine or guanosine accumulation can be underlined. Thus the principal modifications can be grouped into five clusters (Fig. 2): i) central metabolism flux redirection: increasing the ribose-5-phosphate supply; ii) *de novo* pathway deregulation; iii) modifications of *salvage* pathways; iv) alteration of regulators and v) increasing export to the culture media.

7. Concluding remarks

The field of nucleotide and nucleoside production is continuously evolving in parallel with scientific advances (Fig. 3). As a consequence, modern technologies such as rational metabolic and genetic engineering have allowed a significant leap ahead from the former random mutagenesis approaches. Recently, metabolomics, flux balance analyses (FBA) and metabolite flux analyses (MFA) have emerged in the nucleotide production field, with promising results. Hence, in the near future we can expect to find novel approaches that will exploit the benefits of systems biology and synthetic biology together, in the so called systems metabolic engineering (SME) [81]. The potential application of SME has been recently evaluated in industrial microorganisms such as *Aspergillus* and *Pichia* [82] and also in nucleoside-producer organisms such as *Bacillus* [83,84], *Corynebacterium* [85,86] and *Escherichia* [87,88]. So far, no SME approaches have been developed with the objective of improving nucleotide production, although the increasing accessibility to perform wide-omics experiments together with the availability of previously published genome-scale metabolic models for the so called producer organisms [89–91], make this emerging technology a very promising field that will undoubtedly lead to further overcome current production limits.

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