UNIVERSIDAD NACIONAL AUTÓNOMA DE MEXICO

Doctorado en Ciencias Biomédicas

Centro de Ciencias Genómicas

Caracterización fisiológica y bioquímica de mutantes deficientes en la biosíntesis de poliaminas en *Sinorhizobium meliloti* Rm8530

TESIS

Que para optar por el grado de:

DOCTOR EN CIENCIAS

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Cuernavaca, Morelos febrero 2020.

Indicé

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Este trabajo está dedicado a Dalila Sarmiento, quien me ha apoyado y cuidado un largo tiempo. Además de embarcarse conmigo en una gran aventura, de la cual aún queda tanto por descubrir. Gracias por apostar todo junto a mí.

Agradezco a todos los que hicieron posible este trabajo, a mi madre y hermanos. A los Dres. Alejandro García de los Santos y Guillermo Gosset Lagarda, porque siempre ofrecieron excelentes observaciones y comentarios durante mi estancia doctoral. Al Dr. Michael Dunn, de quien aprendí tanto de manera profesional como personal. Gracias por guiarme a lo largo de este trayecto y permitirme infinita libertad en el desarrollo de esta investigación.

A los señores revisores, Dra. Laura Camarena, Dr. Christian Sohlenkamp, Dr. Roberto Arreguín Espinosa de los Monteros y Dra. Guadalupe Espin, por tomarse el tiempo para revisar este manuscrito. A mis compañeros de laboratorio, Alejandra Arteaga y Victor Hernández con quienes compartí tantos experimentos.

Este trabajo fue financiado por DGAPA-PAPIIT IN210114 y IN206317. La obtención del grado fue posible gracias a la beca 574042 de CONACYT.

Abreviaturas

DAP	1,3-diaminopropano
ARN	ácido ribonucleico
DABA	ácido diaminobutírico
cAMP	adenosín monofosfato cíclico
Agm	agmatina
ApCad	aminopropilcadaverina
AbuHSpd	aminobutilhomoespermidina
ADC	arginina descarboxilasa
Cad	cadaverina
CNSpd	carboxinorespermidina
CSpd	carboxiespermidina
HPTLC	cromatografía de capa fina de alta resolución
Spd	espermidina
Spm	espermina
EPS-II	exopolisacárido dos o galactoglucano
EPS-I	exopolisacárido uno o succinoglicano
HSpd	homoespermidina
HSS	homoespermidina sintasa
L-Arg	L-arginina
L-Asp ß-SA	L-aspartato ß-semialdehído
L-Lys	L-lisina
LDC	lisina descarboxilasa
L/ODC	lisina/ornitina descarboxilasa
L-Orn	L-ornitina
NADPH	nicotinamida adenina dinucleótido fosfato reducido
NAD^+	nicotinamida adenina dinucleótido oxidado
NADH	nicotinamida adenina dinucleótido reducido

NSpd	norespermidina
ODC	ornitina descarboxilasa
PLP	piridoxal 5'-fosfato
PAs	poliaminas
Put	putrescina
SAM	*S*-adenosilmetionina

Resumen

Las poliaminas son moléculas ubicuas, alifáticas y policationicas, derivadas de la descarboxilación de diferentes L-aminoácidos. Algunos miembros de este grupo son la espermidina, espermina o putrescina, esta última puede ser sintetizada directa o indirectamente a partir de L-ornitina o L-arginina, respectivamente. Además, dicha diamina es particularmente importante ya que funge como precursor de distintas poliaminas. En bacterias, estos compuestos juegan papeles importantes en diversos procesos biológicos, por ejemplo, crecimiento y/o viabilidad celular, resistencia a estrés, motilidad, patogénesis o formación de biofilm. En rizobias, aunque las poliaminas han demostrado ser importantes en vida libre y simbiosis, sus funciones específicas al igual que su biosíntesis aún deben ser elucidadas. De acuerdo con el genoma de *Sinorhizobium meliloti*, los genes *smc02983* y *sma0680* codifican para dos ornitina descarboxilasas (ODC). En este trabajo, se demostró que una mutante sencilla en *sma0680* (*odc1*) no presenta cambios en su perfil de poliaminas ni defectos fisiológicos con respecto a la cepa parental. Por el contrario, una mutante en *smc02983* (*odc2*) al igual que una doble mutante (*odc1 odc2*) redujeron significativamente su contenido de poliaminas y actividad enzimática. Ensayos con las enzimas purificadas mostraron que ODC2 (SMc02983) exhibe mayor actividad especifica de ODC que ODC1 (SMa0680), además de ser capaz de descarboxilar L-lisina. El bajo contenido de poliaminas en la mutante *odc2* resultó en una menor tasa de crecimiento, mayor sensibilidad a estrés abiótico, menor motilidad, reducción en la producción de exopolisacáridos y una capacidad simbiótica deficiente, mientras su formación de biofilm fue aumentada. En conjunto, estos resultados proporcionan nuevas pistas para comprender los roles que cumplen las poliaminas en rizobias, tanto en vida libre como en asociación con leguminosas.

Abstract

Polyamines are ubiquitous, aliphatic and polycationic molecules, derived from the decarboxylation of different L-amino acids. Some members of this group are spermidine, spermine and putrescine, the latter can be synthesized directly or indirectly from L-ornithine or L-arginine, respectively. This diamine is particularly important since it serves as a precursor of different polyamines. In bacteria, these compounds play important roles in several biological processes, such as cell growth and/or viability, resistance to stress, motility, pathogenesis and biofilm formation. In rhizobias, polyamines have proven to be important in free life and symbiosis, however their specific functions as well as their biosynthesis must still be elucidated. According the *Sinorhizobium meliloti* genome, the *smc02983* and *sma0680* genes encode two ornithine decarboxylases (ODC). In this work, it was shown that a mutant in *sma0680* (*odc1*) does not show differences compared to the parental strain, while a mutant in *smc02983* (*odc2*) as well as a double mutant (*odc1 odc2*), significantly reduced their polyamines content and enzymatic activity. Activity assays with the purified enzymes showed that ODC2 (SMc02983) exhibits greater ODC specific activity than ODC1 (SMa0680), besides being able to decarboxylate L-lysine. The low polyamine content in the *odc2* mutant resulted in a lower growth rate, greater sensitivity to abiotic stress, lower motility, reduced exopolysaccharide production and a poor symbiotic capacity, while biofilm formation was increased. In summary, these results provide new clues to understanding the roles that polyamines play in rhizobia, both in free life and in association with legumes.

1. Introducción

1.1 Poliaminas

Las poliaminas (PAs) son moléculas policatiónicas con dos o más grupos amino, alifáticas y de bajo peso molecular (Tabor & Tabor, 1984; Fujihara, 2009). Las cuales se encuentran ampliamente distribuidas en la naturaleza con excepción de *Trypanosoma cruzi, Toxoplasma gondii* (Algranati, 2010), *Enterococcus faecalis* y *Staphylococcus aureus* (Li et al., 2019), descritos hasta ahora como los únicos organismos auxótrofos de PAs.

Estos compuestos desempeñan importantes roles en la viabilidad y proliferación celular, bajo condiciones normales o de estrés (Fujihara, 2009; Miller-Fleming et al., 2015). En bacterias, las PAs han sido implicadas en numerosas funciones fisiológicas (Shah & Swiatlo, 2008; Michael, 2018). Por ejemplo, la formación de biofilm, motilidad, patogénesis en plantas o animales, crecimiento celular, síntesis de sideróforos, promoción del crecimiento o respuesta al estrés abiótico [ver Tabla 1].

Esta amplia variedad de funciones podría ser resultado de su naturaleza policatiónica que les permite asociarse con diferentes biomoléculas, incluyendo el ARN. Lo cual mejora la expresión de un amplio grupo de genes a nivel de la traducción, incluyendo factores de la trascripción como: RpoS, FecI o Fis. En *Escherichia coli*, este conjunto de genes conforma el denominado “modulón de PAs” (Igarashi & Kashiwagi, 2006; 2015).

Las diaminas presentes en bacterias incluyen putrescina (Put), cadaverina (Cad) y 1,3-diaminopropano (DAP). La primera es producida por casi todas las bacterias, seguida de Cad ampliamente distribuida en proteobacterias, mientras DAP se encuentra esporádicamente en algunos filos (Michael, 2016). La espermidina (Spd) es la triamina más frecuente, aunque diversas bacterias son capaces de sintetizar homoespermidina (HSpd) y norespermidina (NSpd). Con

respecto a las tetraminas, la espermina (Spm) ha sido comúnmente reportada en múltiples especies bacterianas. No obstante, su presencia aun es controversial ya que hasta el momento no se conoce ninguna Spm sintasa bacteriana (Hamana & Matsuzaki, 1992; Michael, 2016; Dunn, 2017).

Tabla 1. Papeles fisiológicos de las poliaminas en bacterias no rizobiales.

Rol biológico	Poliamina y organismo	Referencia
Biofilm	En *Bacillus subtilis* la síntesis de Spd es necesaria para la formación de biofim.	Burrell et al., 2010.
	La Put promueve la expresión de proteínas (Hms) esenciales para la formación de biofilm de *Yersinia pestis*.	Wortham et al., 2010.
	En *Vibrio cholera*, la NSpd es censada por el sistema NspS-MbaA, activando la transcripción de los genes (*vps*) biosínteticos de exopolisacáridos y formación de biofilm, mientras el efecto contrario es desencadenado por Spm.	Cockerell et al., 2014; Sobe et al., 2017.
	La formación de biofilm en *Shewanella oneidensis* y *Synechocystis* PCC 6803 está ligada a la reducción de los niveles de Put y Spd, respectivamente.	Ding et al., 2014; Kera et al., 2018.
	En *Dickeya zeae*, el contenido intracelular de Put es esencial para la formación de biofilm.	Shi et al., 2019.
Estrés	Bajo alta osmolaridad, *E. coli* muestra un activo importe de K^+, el cual es acompañado de la excreción de Put, constituyendo un mecanismo de equilibrio de cargas que mantiene la electroneutralidad y equilibrio iónico intracelular.	Munro et al., 1972.
	La Put y Spd son capaces de unirse a OmpF y OmpC, modificar su carga y tamaño, disminuyendo la permeabilidad de la membrana externa y mejorando la resistencia al estrés ácido y osmótico de *E. coli*.	Iyer et al., 2000.
	En *E. coli*, durante estrés oxidativo la Put y Spd promueven la expresión del factor transcripcional *oxyR* responsable de la expresión de genes de respuesta a este estrés.	Tkachenko & Nesterova 2003.
	La síntesis de Cad es un mecanismo clave en la resistencia a estrés ácido de *E. coli*. El cual consume H^+ provenientes del exterior, conservando el pH fisiológico. Además, la exportación de Cad neutraliza el ambiente extracelular.	Soksawatmaekhin et al., 2004.
	La sobreexpresión de Cad elimina radicales superóxidos e	Kim et al., 2006.

	incrementa la tolerancia a estrés oxidativo en *V. vulnificus*. En *Pseudomonas aeruginosa*, la Spd es capaz de unirse a los lipopolisacáridos, estabilizando y protegiendo la membrana externa contra antibióticos y daño oxidativo. El contenido Put y Spd en *Halomonas BVR* 1 aumenta durante el estrés por metales pesados (Pb). Probablemente constituye un sistema de eliminación de especies reactivas de oxígeno y otros radicales generados en esta condición.	Johnson et al., 2012. Manasi et al., 2017.
Sideróforos	La aminocelina de *Azotobacter vinelandii* contiene Put, la cual también es un precursor de la alcaligina en *Bordetella* spp. La NSpd es un componente estructural de fluvibactina, vulnibactina y vibriobactina de *V. fluvialis, V. vulnificus* y *V. cholerae*, respectivamente. En *B. anthracis,* la Spd es requerida para la síntesis de petrobactina. Diversas bacterias emplean Put, Cad y/o DAP en la biosíntesis de sideróforos de hidroxamato tipo NIS.	Page & von Tigerstrom, 1988; Brickman & Armstrong, 1996. Yamamoto et al., 1993; Okujo et al., 1994; Keating et al., 2000. Oves-Costales et al., 2007. Burrell et al., 2012.
Motilidad	La Put y Cad fungen como moléculas quimiotácticas en *P. aeruginosa* y *P. putida*. En *Proteus mirabilis*, la Put funge como señal para el desarrollo del swarming, mientras en *E. coli*, el mismo tipo de motilidad requiere de Spd. La motilidad independiente de flagelo en *Acinetobacter baumannii* requiere DAP. En *D. zeae*, la motilidad tipo swimming y swarming son dependientes de Put.	Taguchi et al., 1997; Corral-Lugo et al., 2016. Sturgill et al., 2004; Kurihara et al., 2009. Skiebe et al., 2012. Shi et al., 2019.
Crecimiento vegetal	La Spd producida por *B. subtilis* OKB105 induce la expresión de genes de expancina e inhibe la síntesis de etileno, promoviendo el crecimiento de tabaco.	Xie et al., 2014.
Patogénesis	En *Shigella*, niveles elevados de Spd se asocian con una mayor supervivencia durante la infección, mientras la falta Cad aumenta la su patogenicidad en los tejidos del huésped. La virulencia de *Y. pestis* requiere Put y/o Spd. La Cad y Spd están asociadas con la colonización y desarrollo de neumonía en ratón por *Streptococcus pneumoniae*.	Maurelli et al., 1998; Barbagallo et al., 2011. Wortham et al., 2010. Shah et al., 2011.

	En *Salmonella enterica serovar* Typhimurium, la invasión, supervivencia intracelular, muerte de *Caenorhabditis elegans* e infección sistémica en ratón por fiebre tifoidea requiere Put y/o Spd. *P. mirabilis* exhibe una invasión a células uroteliales dependiente de Put. La inactivación de la síntesis y transporte de Put de *D. zeae*, reduce significativamente su virulencia en arroz.	Jelsbak et al., 2012. Kurihara et al., 2013. Shi et al., 2019.
Estructural	Los peptidoglicanos de *Selenomonas ruminantium*, *Veillonella alcalescens* y *V. parvula* contienen Cad o Put. En *Anaerovibrio lipolytica*, la Spd y Cad constituyen parte del peptidoglicano. La Put está asociada a los lipopolisacáridos de *P. mirabilis*.	Kamio et al., 1981; 1987; Kamio & Nakamura, 1987. Hirao et al., 2000. Vinogradov & Perry, 2000.
Crecimiento celular	La Spd es esencial para el crecimiento de *P. aeruginosa*, *Campylobacter jejuni* y *Borrelia burgdoferi*. Mientras para *Ralstonia solanacearum* su crecimiento requiere Put. Aunque muchas bacterias no tienen un requerimiento absoluto de PAs, la reducción de su contenido intracelular afecta su crecimiento en diferentes niveles.	Nakada & Itoh, 2003; Hanfrey et al., 2011; Bontemps-Gallo et al., 2018; Lowe-Power et al., 2018; Michael, 2018.

1.2 Biosíntesis de poliaminas

Las PAs son derivadas de la descarboxilación de L-ornitina (L-Orn), L-arginina (L-Arg) y L-lisina (L-Lys), mediada por enzimas dependientes de piridoxal 5'-fosfato (PLP), las cuales exhiben el plegamiento alanina racemasa o aspartato aminotransferasa. Una de estas enzimas es la ornitina descarboxilasa (ODC; EC 4.1.1.17), que sintetiza Put a partir de L-Orn, la cual en *E. coli* puede inducirse por acidez (*speF*) o expresarse constitutivamente (*speC*) [Fig. 1] (Burrell et al., 2010).

La diamina Put es el precursor de Spd y Spm, la síntesis de la primera requiere la adición de un grupo aminopropil proveniente de la descarboxilación de *S*-adenosilmetionina (SAM), mientras la segunda emplea dos. Dichas reacciones son catalizadas por las sintasas de Spd (EC 2.5.1.16) y Spm (EC 2.5.1.22), respectivamente (Peeg, 1986; Shah & Swiatlo, 2008).

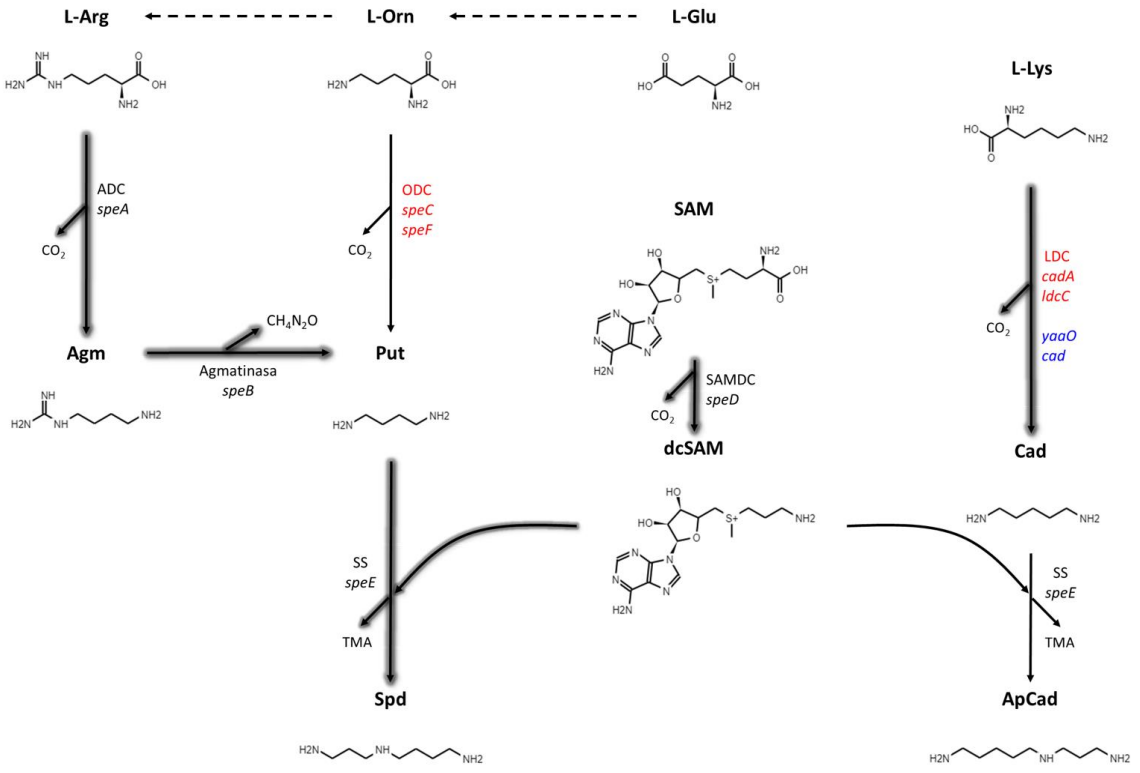

Figura 1. Ruta de síntesis de PAs en *E. coli* y *B. subtilis*. El diagrama muestra la vía caracterizada en *E. coli*, en el cual las flechas sombreadas indican las reacciones presentes en *B. subtilis.* Los genes marcados en negro corresponden a los presentes en ambos organismos, mientras en rojo y azul se muestran los genes propios de *E. coli* y *B. subtilis*, respectivamente. La ruta biosintética en *E. coli* contiene 2 ODC (codificadas por *speC* y *speF*), contrario a *B. subtilis* que carece de esta enzima. Las flechas punteadas resumen la biosíntesis de L-Arg. Abreviaturas no mostradas en el texto: SAMDC (*S*-adenosilmetionina descarboxilasa), dcSAM (*S*-adenosilmetionina descarboxilado), SS (Spd sintasa) y TMA (tiometiladenosina). Revisado en: Bowman et al., 1973; Igarashi et al., 1986; Yamamoto et al., 1997; Sekowska et al., 1998; Shah & Swiatlo, 2008.

Alternativamente, la Put puede ser sintetizada mediante las reacciones de arginina descarboxilasa (ADC; EC 4.1.1.19) y agmatinasa (EC 3.5.3.11) [Fig. 1]. En *B. subtilis* y *Synechocystis* sp., esta vía indirecta es la única ruta biosíntetica de PAs (Sekowska et al., 1998; Kera et al., 2018), mientras en organismos como *P. aeruginosa* PAO1 la Put puede generarse mediante las agmatina deiminasa (*aguA*; EC 3.5.3.12) y *N*-carbamoilputrescina amidohidrolasa (*aguB*; EC 3.5.1.53), que catalizan la conversión de agmatina (Agm) a Put a través del intermediario *N*-carbamoilputrescina (Nakada & Itoh, 2003).

La Cad es sintetizada de L-Lys por acción de la lisina descarboxilasa (LDC; EC 4.1.1.18) y por las descarboxilasas bifuncionales denominadas lisina/ornitina descarboxilasa (L/ODC; EC 4.1.1.18/EC 4.1.1.17) presentes en *S. ruminantium*, *V. vulnificus* y *Sinorhizobium meliloti* (Yamamoto et al., 1997; Takatsuka et al., 2000; Burrell et al., 2010; Becerra-Rivera et al., 2018).

En *E. coli*, la Cad puede fungir como sustrato alternativo de la Spd sintasa y contender contra el déficit de PAs, mediante la síntesis de la triamina compensatoria aminopropilcadaverina (ApCad) [Fig. 1] (Bowman et al. 1973; Igarashi et al., 1986). Esta última, en conjunto con la aminobutilcadaverina (AbuCad), HSpd y aminobutilhomoespermidina (AbuHSpd) están presentes en un grupo de α- y β-proteobacterias comúnmente llamado rizobias (Fujihara, 2009; Weir, 2016).

1.3 Rizobias

Estas bacterias comprenden ~100 especies, incluyendo tanto fijadoras como no fijadoras de nitrógeno. Las cuales se distribuyen en géneros como: *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Bradyrhizobium* o A*grobacterium*. En ambientes limitantes de nitrógeno, ciertos organismos fijadores pueden entablar una relación simbiótica con las raíces de plantas leguminosas y reducir el nitrógeno atmosférico en órganos especializados denominados nódulos (Soto et al., 2006, Weir, 2016, Poole et al., 2018).

La fijación de nitrógeno requiere la diferenciación de las bacterias a bacteroide, la cual se da a través de un continuo intercambio de señales entre planta y endosimbiontes. El proceso inicia cuando los flavonoides presentes en los exudados de la raíz activan la expresión de los genes de nodulación bacterianos que sintetizan y secretan de factores Nod (lipoquitooligosacaridos), los cuales son reconocidos por la planta. Dichas moléculas en conjunto con señales microbianas

adicionales, permiten que las bacterias ingresen a la raíz a través del hilo de infección, estructura que crece hacia la corteza donde eventualmente se desarrollará el nódulo (Leigh et al., 1985; Soto et al., 2006).

1.4 *Sinorhizobium meliloti*

Es una bacteria Gram negativa capaz de sintetizar el exopolisacárido succinoglicano (EPS-I), que al igual que los factores Nod es esencial para una simbiosis efectiva con diversas especies de los géneros *Medicago*, *Melilotus* y *Trigonella*. No obstante, algunas cepas *S. meliloti* son capaces de producir un segundo exopolisacárido llamado galactoglucano (EPS-II), el cual previene los defectos simbióticos por ausencia del EPS-I en *M. sativa* (Leigh et al., 1985; Glazebrook & Walker, 1989; Lehman & Long, 2013).

1.5 Poliaminas en *S. meliloti*

En comparación a otras rizobias, *S. meliloti* cuenta con una vía de síntesis PAs más compleja, resultando en un mayor contenido de PAs que incluye Put, Spd, HSpd, DAP y NSpd (Becerra-Rivera et al., 2018; Becerra-Rivera & Dunn, 2019). En *S. meliloti* 1021, las ADC y ODC son codificadas por *sma0682* y *sma0680*, ubicados en un operón del plásmido pSymA (Schlüter et al., 2013). Probables antiportadores de Agm/Arg (*sma0684*) y Put/Orn (*sma0678*) están ubicados de forma adyacente a estas descarboxilasas (Dunn, 2017), dicha organización es típica en las descarboxilasas inducibles por acidez (Kanjee & Houry, 2013).

Esta α-proteobacteria también presenta una descarboxilasa bifuncional (L/ODC), la cual es codificada por el gen cromosomal *smc02983* (Becerra-Rivera et al., 2018). Dicha enzima, probablemente es clave en la biosíntesis de PAs en rizobias, ya que a diferencia de la ODC, ortólogos de esta L/ODC están presentes en especies de *Rhizobium*, *Sinorhizobium, Mesorhizobium* y *Bradyrhizobium* (Becerra-Rivera & Dunn, 2019).

En *S. meliloti*, la Put puede emplearse para generar HSpd o Spd [Fig. 2] (Dunn, 2017). Contrario a esta última, la HSpd es sintetizada en una sola reacción mediada por la homoespermidina sintasa (HSS; EC 2.5.1.44), cuyo gen se localiza en el cromosoma de la bacteria. La reacción inicia con la desaminación oxidativa de una molécula de Put, un mecanismo dependiente de nicotinamida adenina dinucleótido (NAD^+). El semialdehído resultante reacciona con una segunda Put produciendo un intermediario de imina que es reducido por el NADH, sintetizando HSpd (Shaw et al. 2010; Shaw, 2011).

La HSS es una proteína bien distribuida en α-, γ- y δ-proteobacterias, particularmente en patógenos como *Legionella pneumophila*, *P. aeruginosa* o *Brucella sp*. (Shaw, 2011; Krossa et al., 2016). Y es capaz de usar diversos sustratos como Spd + Put, Spd + Cad o Put + Cad, sintetizando DAP y HSpd, AbuCad y DAP o solo AbuCad, respectivamente (Böttcher et al., 1994; Fujihara, et al. 1995; Shaw et al., 2010; Shaw, 2011; Krossa et al., 2016).

La síntesis de Spd en *S. meliloti* es catalizada mediante una ruta "alternativa" descrita en *V. cholerae*, la cual no involucra una Spd sintasa o SAM descarboxilado (Lee et al., 2009; Dunn, 2017). En su lugar emplea L-aspartato ß-semialdehído (L-Asp ß-SA) y las enzimas carboxinorespermidina (CNSpd) deshidrogenasa (SMb21630; EC 1.5.1.43) y CNSpd descarboxilasa (SMb21631; EC 4.1.1.96), las cuales catalizan dos reacciones secuenciales para producir Spd de Put [Fig. 2] (Shaw et al., 2010; Hanfrey et al., 2011).

Ambas enzimas están codificadas en el plásmido pSymB y también participan en síntesis de NSpd. La CNSpd deshidrogenasa media una reacción dependiente NADPH y transfiere un grupo aminopropil donado por L-Aspß-SA a Put o DAP, formando carboxiespermidina (CSpd) o CNSpd, respectivamente. Los cuales son descarboxilados en una segunda reacción dependiente de PLP, para finalmente producir Spd o NSpd (Lee et al., 2009; Hanfrey et al., 2011).

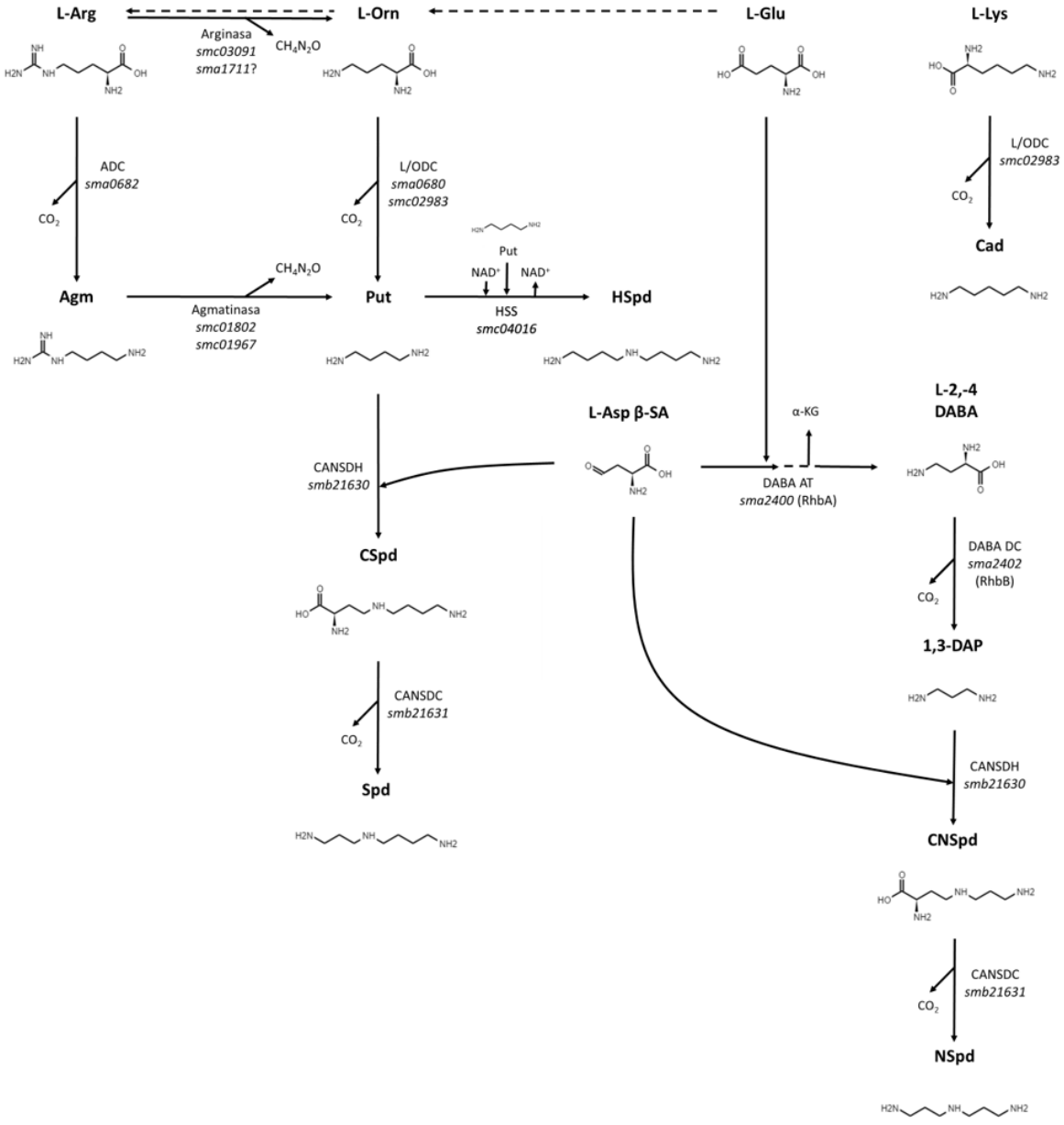

Figura 2. Vía de síntesis de PAs en *S. meliloti* 1021. Esta ruta está basada en las anotaciones presentes en Kyoto Encyclopedia of Genes and Genomes y la similitud de secuencia con *V. cholerae*. Las flechas punteadas resumen la biosíntesis de arginina. Abreviaturas no mostradas en el texto: L-Glu (L-glutamato), α-KG (α-cetoglutarato), CANSDH (CNSpd deshidrogenasa) y CANSDC (CNSpd descarboxilasa). Modificado de: Becerra-Rivera et al., 2018.

Los precursores de la biosíntesis de NSpd son sintetizados por las enzimas ácido diaminobutírico (DABA) aminotransferasa (RhbA; EC 2.6.1.76) y DABA

descarboxilasa (RhbB; EC 4.1.1.86) [Fig. 2]. Estas constituyen dos reacciones continuas, la primera es reversible y liga el grupo amino del L-glutamato a L-Aspß-SA, formando ácido L-2,4-diaminobutírico que es descarboxilado a DAP por DABA descarboxilasa (Lee et al., 2009; Michael, 2016). Ambas enzimas han sido caracterizadas en la síntesis del sideróforo rizobactina en *S. meliloti* 1021 (Lynch et al., 2001).

1.6 Antecedentes

En rizobias, diversas investigaciones han demostrado la importancia de las PAs en múltiples procesos fisiológicos. Por ejemplo, en *R. leguminosarum* bv. viciae 3841 (Shaw, 2011) y *A. tumefaciens* C58 (Kim et al., 2016; Wang et al., 2016) se observó que la inhibición o inactivación de la ODC disminuye fuertemente los niveles intracelulares de PAs, resultando en una reducción significativa de su crecimiento celular. Asociado a estos defectos Wang et al. (2016) reportó mayor síntesis de celulosa y formación de biofilm. En ambos organismos todos los fenotipos fueron restaurados por Put exógena.

Además del crecimiento o formación de biofilm, la motilidad es otro proceso alterado por los niveles de PAs. El desplazamiento sobre superficies (swarming) es abatido en una mutante en HSS de *R. etli* CNPAF512 (Braeken et al., 2008), este fenotipo también fue descrito en una mutante en *rhbA* (DABA aminotransferasa) de *S. meliloti* 1021. De acuerdo con el transcriptoma de *fadD*, una mutante de *S. meliloti* con mayor swarming, la expresión de algunos genes como *rhbB* (DABA descarboxilasa) incrementó considerablemente (Nogales et al., 2010). Como se mencionó anteriormente, ambos genes (*rhbA* y *rhbB*) están involucrados en la biosíntesis de DAP [Fig. 2] y rizobactina (Lynch et al., 2001).

Las PAs también han sido implicadas en mecanismos de resistencia a estrés abiótico. En *S. fredii* P220, los niveles de HSpd son incrementados o reducidos al cultivarla en condiciones acidas o salinas, respectivamente. Este

compuesto ha sido propuesto como parte de un mecanismo de equilibrio de cargas intracelulares (Fujihara & Yoneyama, 1993). Por otra parte, una mutante de HSS en *R. tropici* CIAT899 presenta poca tolerancia a estrés salino y reducción de la biomasa de los nódulos formados en *Phaseolus vulgaris* (López-Gómez et al., 2016b). De manera consistente, la HSpd es la poliamina más abundante en nódulos formados por rizobias como *S. meliloti* o *R. tropici* (Fujihara, 2009; López-Gómez et al., 2014; López-Gómez et al., 2016a; 2016b).

De acuerdo con Vassileva e Ignatov (1999), la adición de Put, Spd o Spm exógenas incrementa la biomasa y fijación de nitrógeno en nódulos formados por *R. galagae* HAMBI 540 en *Galega orientalis*. Estas moléculas posiblemente son usadas por los microsimbiontes como fuente de energía, además de promover la captación de malato en los bacteroides como en la simbiosis *B. japonicum* USDA 110-*Glycine max* (Whitehead et al., 2001).

En la relación simbiótica *S. meliloti* 1021-*M. sativa,* recientemente se demostró que la adición de triaminas (Spd y Spm) incrementa la biomasa de raíz y brotes, la actividad de nitrogenasa e induce la acumulación de estas PAs en hojas (López-Gómez et al., 2017). Al igual que en soya y *M. truncatula*, los autores reportaron cambios en los niveles de brassinosteroides *(*Terakado-Tonooka & Fujihara, 2008; López-Gómez et al., 2016a; López-Gómez et al., 2017). En resumen, las PAs influyen en la fisiología de numerosos organismos, incluidas las rizobias. Por ende, elucidar la vía de síntesis de PAs en *S. meliloti*, mediante la mutagénesis de pasos clave, así como su caracterización fisiológica, proporcionará una mejor comprensión de la biología de este organismo.

1.7 Hipótesis

En *S. meliloti* 1021, la (i) transcripción y (ii) actividad de agmatinasa (SMc01802) es sumamente baja (Díaz et al., 2011; Dunn, datos no publicados), (iii) incluso su sustrato (Agm) no se ha podido detectar entre sus PAs

intracelulares. Por el contrario, (iv) la expresión y actividad de arginasa (SMc03091) es muy alta (Arteaga et al., datos no publicados), sintetizando L-Orn para la ODC (SMa0680) y L/ODC (SMc02983). Por lo tanto, hipotetizamos que **la biosíntesis de Put en *S. meliloti* ocurre a través de SMa0680 y/o SMc02983 y no mediante la vía de ADC/Agmatinasa.**

1.8 Objetivo general

Caracterizar los efectos fisiológicos de la inactivación de la Odc y L/Odc en *Sinorhizobium meliloti* Rm8530.

1.9 Objetivos particulares

Generar mutantes simples (*sma0680* y *smc02983*) y una mutante doble (*sma0680 smc02983*), mediante mutagénesis dirigida.

Confirmar los fenotipos de las mutantes por ensayos enzimáticos de descarboxilación de aminoácidos.

Analizar el crecimiento de las mutantes en condiciones normales y bajo estrés abiótico.

Realizar un análisis del contenido de poliaminas mediante HPTLC.

Evaluar la producción de exopolisacáridos, motilidad y formación de biofilm de una mutante deficiente de poliaminas.

Determinar la importancia de la biosíntesis de poliaminas en la simbiosis *S. meliloti-M. sativa*.

Purificar ambas descarboxilasas y determinar sus especificidades de sustrato (L-Lys, L-Orn o L-Arg).

2. Resultados

MICROBIOLOGY

RESEARCH ARTICLE

Becerra-Rivera *et al.*, *Microbiology* 2018;164:600–613

DOI 10.1099/mic.0.000615

MICROBIOLOGY SOCIETY

Polyamines are required for normal growth in *Sinorhizobium meliloti*

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Abstract

Polyamines (PAs) are ubiquitous polycations derived from basic L-amino acids whose physiological roles are still being defined. Their biosynthesis and functions in nitrogen-fixing rhizobia such as *Sinorhizobium meliloti* have not been extensively investigated. Thin layer chromatographic and mass spectrometric analyses showed that *S. meliloti* Rm8530 produces the PAs, putrescine (Put), spermidine (Spd) and homospermidine (HSpd), in their free forms and norspermidine (NSpd) in a form bound to macromolecules. The *S. meliloti* genome encodes two putative ornithine decarboxylases (ODC) for Put synthesis. Activity assays with the purified enzymes showed that ODC2 (SMc02983) decarboxylates both ornithine and lysine. ODC1 (SMa0680) decarboxylates only ornithine. An *odc1* mutant was similar to the wild-type in ODC activity, PA production and growth. In comparison to the wild-type, an *odc2* mutant had 45 % as much ODC activity and its growth rates were reduced by 42, 14 and 44 % under non-stress, salt stress or acid stress conditions, respectively. The *odc2* mutant produced only trace levels of Put, Spd and HSpd. Wild-type phenotypes were restored when the mutant was grown in cultures supplemented with 1 mM Put or Spd or when the *odc2* gene was introduced *in trans*. *odc2* gene expression was increased under acid stress and reduced under salt stress and with exogenous Put or Spd. An *odc1 odc2* double mutant had phenotypes similar to the *odc2* mutant. These results indicate that ODC2 is the major enzyme for Put synthesis in *S. meliloti* and that PAs are required for normal growth *in vitro*.

INTRODUCTION

Polyamines (PAs) are low molecular weight organic compounds with two or more amino groups that are positively charged at neutral pH [1]. With few exceptions, PAs are ubiquitous in all organisms and have important roles in processes as diverse as growth, stress resistance and the regulation of transcription and translation in both eukaryotes and prokaryotes [2–5]. In contrast to their essential functions in eukaryotes and archaea, the physiological roles of PAs in bacteria are less clearly defined. In prokaryotes, PAs are involved in biofilm formation, stress resistance, motility, pathogenesis and growth [6–12]. This diversity of functions might explain the presence of the more varied PA repertoire in bacteria [5].

Diamines found in bacteria include putrescine (Put), cadaverine (Cad) and 1,3-diaminopropane (DAP) (Fig. 1). Put is produced by nearly all bacteria, Cad is common in Proteobacteria and DAP is found sporadically in diverse phyla. Spermidine (Spd) is the most commonly found triamine, though bacteria may produce the related homospermidine (HSpd) and/or, less commonly, norspermidine (NSpd) [2, 5, 13].

Put can be made by the decarboxylation of L-ornithine (Orn) by Orn decarboxylase (ODC; EC 4.1.1.17) or of L-arginine (Arg) by Arg decarboxylase (ADC; EC 4.1.1.19). The ADC reaction produces agmatine (Agm), which is converted to Put by agmatinase (SpeB; EC 3.5.3.11). Cad is produced by lysine decarboxylase (LDC; EC 4.1.1.18) acting on

Received 12 November 2017; Accepted 19 January 2018

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Keywords: *S. meliloti*; polyamines; putrescine; spermidine; homospermidine; norspermidine; ornithine decarboxylase.

Abbreviations: ADC, arginine decarboxylase; Agm, agmatine; Arg, L-arginine; L-Asp β-SA, L-aspartate β-semialdehyde; Cad, cadaverine; Cb, carbenicillin; CID, collision-induced dissociation; DAP, 1,3-diaminopropane; DNS, dansyl group; DNSCl, dansyl chloride; DNS-PA, dansyl-polyamine; FT-ICR, Fourier-transform ion cyclotron resonance; Gm, gentamicin; Gus, β-glucuronidase; *gusA*, gene encoding β-glucuronidase; HPTLC, high-performance thin layer chromatography; HSpd, homospermidine; Km, kanamycin; LB, Luria-Bertani; Lys, L-lysine; LDC, lysine decarboxylase; MALDI, matrix-assisted laser desorption/ionisation; MMS, minimal medium succinate-ammonium; MMS-acid, MMS medium, pH 5.5; MMS-Salt, MMS medium with 0.3 M NaCl; MS, mass spectrometry; MS/MS, tandem mass spectrometry; μ, generations h^{-1}; Nm, neomycin; NSpd, norspermidine; OD_{600}, optical density at 600 nm; ODC, ornithine decarboxylase; *odc1*, gene encoding ornithine decarboxylase SMa0680; *odc2*, gene encoding lysine/ornithine decarboxylase SMc02983; Orn, L-ornithine; PA, polyamine; Put, putrescine; PY, peptone-yeast extract; Sp, spectinomycin; Spd, spermidine; Spm, spermine; Sm, streptomycin; TCA, trichloroacetic acid; TSS, transcriptional start site.

One supplementary table and one supplementary figure are available with the online version of this article.

L-lysine (Lys). Some decarboxylases have activity with both Lys and Orn as substrates [5, 13, 14].

In *Sinorhizobium meliloti* and other nitrogen-fixing rhizobia, work on PAs has focused on their identification and quantification during free-living growth [15, 16], changes in their levels *in nodulo* when host plants were subjected to abiotic stress, or determining the effects of exogenous polyamines on symbiosis [12, 17–21]. A few studies using biochemical and genetic approaches have shown species-dependent requirements for different PAs for growth, biofilm formation and motility in rhizobia [11, 22–25].

Three studies with different strains of *S. meliloti* grown in minimal medium show that its free PA fraction invariably contains Put, Spd and HSpd, with Cad found in two of the studies [15, 16, 19]. Hamana *et al.* [16] assayed for, but did not detect, Agm, spermine (Spm), NSpd or DAP in *S. meliloti* IAM 12611. PAs in *S. meliloti* 1021 have been reported only for cultures grown in rich medium, where Put, Spd, HSpd and Spm were detected [19]; the latter probably originates from Spm present in the tryptone and yeast extract components of the medium [14]. In contrast to other rhizobia, the genome sequence of strain 1021 indicates that it is able to synthesize NSpd, which has not been reported in rhizobia, and possesses an 'alternative' pathway for Spd synthesis [14]. The strain 1021 genome encodes three putative basic amino acid decarboxylases. *sma0682* is annotated as a 'decarboxylase (lysine, ornithine, arginine)' but its genomic context and sequence suggest that it encodes an ADC. We predicted that *sma0680* (annotated as 'amino acid (ornithine, lysine, arginine) decarboxylase') encodes an ODC: we denominate this gene and enzyme as *odc1* and ODC1, respectively. *smc02983* is annotated as an 'ornithine, DAP, or arginine decarboxylase' but its product has sequence characteristics that suggest it is able to decarboxylate both lysine and ornithine [14, 26]. We refer here to the *smc02983* gene and protein product as *odc2* and ODC2, respectively (Fig. 1).

Because *S. meliloti* has multiple enzymes catalysing the conversion of *N*-acetylornithine to Orn during Arg biosynthesis [27] and is also able to produce Orn with an inducible arginase [14, 26], we hypothesized that Orn is the major precursor for Put synthesis in this organism [27]. If this is correct, then ODC1 and/or ODC2 should be the key enzyme(s) for the synthesis of Put and the PAs derived from it (Fig. 1). The work presented here shows that ODC2 is responsible for synthesizing the majority of the Put produced by *S. meliloti* and that PAs are important for normal growth in minimal medium cultures with and without abiotic stress.

METHODS

Bacterial strains, plasmids and culture growth conditions

Bacterial strains and plasmids are listed in Table 1. PY and LB complex media, and MMS minimal medium with

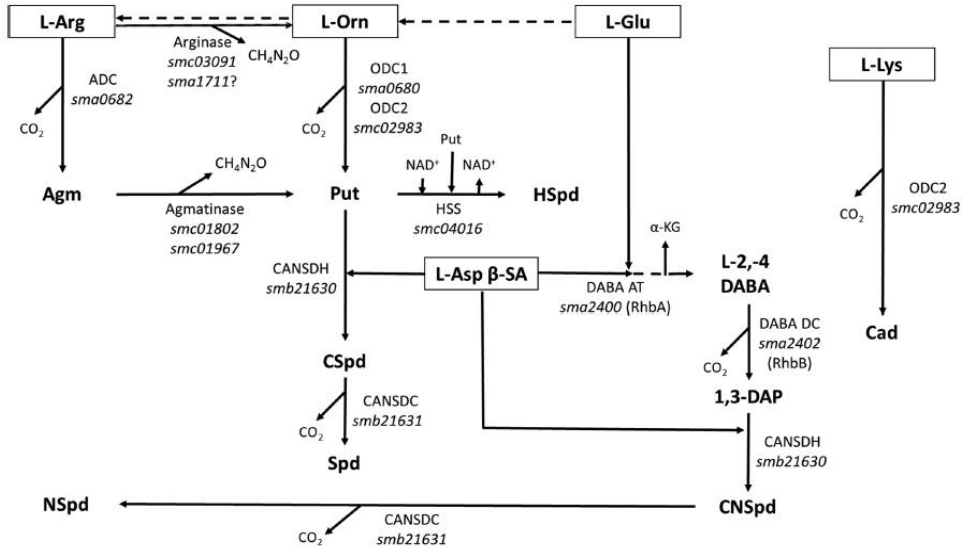

Fig. 1. Predicted polyamine synthesis pathways in *S. meliloti* Rm8530. Abbreviations not described in the text: α-KG, α-ketoglutarate; CNSpd, carboxynorspermidine; CANSDC, CNSpd decarboxylase; CANSDH, CNSpd dehydrogenase; CSpd, carboxyspermidine; DABA, diaminobutyric acid; DABA AT, DABA aminotransferase; DABA DC, DABA decarboxylase; L-Glu, L-glutamate; HSS, homospermidine synthase. Modified from [14].

succinate and NH_4Cl as carbon and nitrogen sources, respectively, were described previously [27]. Salt stress was caused by growing cultures in MMS-salt medium, where NaCl was added to MMS to a final concentration of 0.3 M before adjusting the pH to 6.8 and autoclaving. For growth under acidic stress, MMS-acid medium was prepared by adjusting MMS medium to pH 5.5 (rather than 6.8) before autoclaving. PA and amino acid supplements were prepared as 0.5 M stocks, adjusted to pH 6.8 (for use in MMS and MMS-salt) or pH 5.5 (for use in MMS-acid) and filter sterilized. To grow *S. meliloti* strains, cells from 3 day PY plates with appropriate antibiotics were used to inoculate 3 ml of liquid PY containing the same antibiotics and incubated at 200 r.p.m., 30 °C. After 24 h, 1 ml of these cultures was used to inoculate 50 ml of PY containing one-half of the normal concentration of antibiotics, in 125 ml baffled flasks. These cultures were incubated as above for 24 h, harvested by centrifugation at 5500 ***g*** for 5 min, the cells washed twice in MMS and resuspended to an OD_{600} of approximately 1.5 in MMS. These suspensions were used to inoculate the desired minimal medium without antibiotics to an initial OD_{600} of 0.05: for transcriptional fusion and PA analyses, respectively, the medium:baffled flask volume ratios were 50 : 125 ml and 100 : 250 ml. Cultures were grown with agitation at 200 r.p.m. at 30 °C and growth was monitored at 600 nm. Specific growth rates (μ, generations h^{-1}) were calculated [28] from culture OD_{600} values obtained between 4 and 12 h under non-stress conditions and 4 to 24 h under stress conditions. When required, antibiotics were used at the following concentrations (μg ml^{-1}): carbenicillin (Cb), 50; gentamicin (Gm), 15; kanamycin (Km), 50; neomycin (Nm), 60; spectinomycin (Sp), 100; streptomycin (Sm), 200.

DNA manipulations

Standard protocols were used to grow *E. coli* and for DNA isolation, restriction digests, cloning and transformation

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
***E. coli* strains**		
BL21(DE3)	Strain for protein expression	Invitrogen
DH5α	Cloning strain	Laboratory collection
JM109	Cloning strain	Laboratory collection
***S. meliloti* strains**		
Rm8530	*S. meliloti* 1021 *expR*$^+$, Sm^r	M. Soto, Estación Experimental del Zaidín, CSIC, Granada, Spain
8530 *odc1*	Rm8530 *sma0680*::*loxP* Sp *odc1* null mutant, Sm^r Sp^r	This study
8530 *odc2*	Rm8530 *smc02983*::*loxP* Sp *odc2* null mutant, Sm^r Sp^r	This study
8530 *odc1 odc2*	Rm8530 *sma0680*::*loxP smc02983*::*loxP* Sp *odc1 odc2* null double mutant, Sm^r Sp^r	This study
8530 odc2(pBB5)	Rm8530 *smc02983*::*loxP* Sp *odc2* null mutant containing plasmid pBB5, Sm^r Sp^r Gm^r	This study
8530 odc2(pBB5-odc2)	Rm8530 *smc02983*::*loxP* Sp *odc2* null mutant complemented with the *odc2* gene *in trans*, Sm^r Sp^r Gm^r	This study
Plasmids		
pBB5	Broad-host-range vector pBBR1MCS-5, Gm^r	[43]
pBB5-odc2	pBBR1MCS-5 containing *smc02983* with native promoter and terminator regions, Gm^r	This study
pBBMCS-53	Δ*placZ* pBBR1MCS-5 derivative with promoterless *gusA*, Gm^r	[32]
pBB53odc1::gusA	Transcriptional *sma0680*::*gusA* fusion in pBBMCS-53	This study
pBB53odc2::gusA	Transcriptional *smc02983*::*gusA* fusion in pBBMCS-53	This study
pCRodc1	Rm8530 genome region containing *odc1* cloned in pTopo	This study
pCRodc2	Rm8530 genome region containing *odc2* cloned in pTopo	This study
pKodc1	Rm8530 genome region containing *odc1* cloned in pK18mobsacB	This study
pKodc2	Rm8530 genome region containing *odc2* cloned in pK18mobsacB	This study
pK18mobsacB	Broad-host range gene replacement vector, Km^r	[44]
pKodc1::loxSp	odc1::loxP Sp fragment cloned in pK18mobsacB	This study
pKodc2::loxSp	odc2::loxP Sp fragment cloned in pK18mobsacB	This study
pMS102loxSp17	Source of the loxP Sp interposon, Sp^r	[45]
pRK2013	Helper plasmid, Km^r	[46]
pET-Sumo	Expression vector for production of 6His-Sumo-tagged proteins, Km^r	Invitrogen
pSumo-odc1	pET-Sumo containing the cloned Rm8530 *odc1* gene	This study
pSumo-odc2	pET-Sumo containing the cloned Rm8530 *odc2* gene	This study
pTopo	pCR2.1Topo vector for cloning PCR products, Km^r	Invitrogen
pTZ57R/T	InsTAclone vector for cloning PCR products, Ap^r (Cb^r)	Thermo
pBBRMCre	Plasmid used for deleting the loxP Sp interposon inserted in *smc0680*	[30]

[29]. Bacterial conjugations were performed as described previously [27]. High-stringency DNA hybridizations were done with a DIG-High Prime DNA Labeling kit (Roche).

PCR amplifications

DNA sequences were obtained from GenBank (www.ncbi.nlm.nih.gov/gene/). Primers (Table S1, available in the online version of this article) were used in PCR reactions with Accuprime Taq DNA polymerase (Invitrogen) to clone genes for the construction of transcriptional fusions or whose products were to be overexpressed and purified, or with Dream Taq PCR master mix (Thermo) for other purposes. PCR cycling programs included a denaturing step at 95 °C for 1 min followed by 30 cycles of 95 °C for 1 min, 56 °C for 1 min and 72 °C for a time appropriate for the length of the DNA being amplified. A final elongation step was made at 72 °C for 10 min. For use in cloning, PCR products were purified with a commercially available kit.

Recombinant protein purification

S. meliloti genes *odc1* and *odc2* were amplified by PCR (Table S1) and cloned in pET Sumo to generate plasmids pSumo-odc1 and pSumo-odc2 (Table 1). These plasmids were used to overexpress the corresponding 6His-Sumo tagged protein products in *E. coli* BL21(DE3). For overexpression and purification, strain BL21(DE3) transformed with either pSumo-odc1 or pSumo-odc2 were grown in 100 ml LB Km at 37 °C, 200 r.p.m. to an OD_{600} of 0.4, IPTG was added to a final concentration of 1 mM and incubation continued for 8 and 14 h, respectively. 6His-Sumo tagged proteins were purified using Ni-NTA resin (Invitrogen) under hybrid conditions following the manufacturer's protocol.

Mutant construction

Mutants of *S. meliloti* Rm8530 were constructed by the insertional inactivation of genes as described previously [27]. Briefly, genome regions encoding *odc1* and *odc2* were amplified by PCR (Table S1) and cloned into pCR 2.1-Topo to produce plasmids pCRodc1 and pCRodc2 (Table 1). Following verification by restriction enzyme analysis, the inserts from pCRodc1 and pCRodc2 were excised with *Sma*I/*Xba*I and *Sal*I, respectively, and inserted into suicide vector pK18mobsacB cut with *Sma*I/*Xba*I or *Xho*I to give plasmids pKodc1 and pKodc2 respectively. The loxP Sp cassette from pMS102loxSp17 was ligated into the *Bam*HI and *Sal*I sites of the genes cloned in pKodc1 and pKodc2, respectively, generating plasmids pKodc1 :: loxSp and pKodc2 :: loxSp (Table 1). These constructs were introduced into *S. meliloti* Rm8530 by triparental mating using *E. coli* DH5α/pRK2013 as the helper. A Sp^r Nm^r single recombinant obtained from each mating was spread on PY containing Sm, Sp and 12 % sucrose to allow the selection of the 8530 *odc1* and 8530 *odc2* mutants (Table 1). The 8530 *odc1 odc2* double mutant was constructed in two steps. First, the loxP Sp interposon in the *odc1* gene in the 8530 *odc1* mutant was deleted by introducing plasmid pBBRMCre, which expresses the loxP-specific Cre recombinase, into the mutant. The desired loxP Sp deletion and pBBRMCre plasmid-cured strain was selected by screening for the Sm^r Sp^s and Sm^r Gm^s phenotypes, respectively [30]. In the second step, plasmid pKodc2 :: loxSp was introduced into the 8530 *odc1* loxSp-deleted mutant to obtain the 8530 *odc*1 *odc2* double mutant by selection for sucrose sensitivity. The correct construction of the mutants was confirmed by Southern hybridization.

Genetic complementation of the 8530 *odc2* mutant

To test genetic complementation of the *odc2* mutant, we excised the *Eco*RI fragment from pCRodc2, which contains the *odc2* gene with its native promoter and terminator regions, and introduced it into pBB5 to give plasmid pBB5-odc2. This plasmid, or pBB5 without an insert, was introduced into the 8530 *odc2* mutant by triparental mating.

Basic amino acid decarboxylase assays

The radiochemical assay for determining ODC activity in intact cells was modified from that of Romano *et al.* [31]. Cells from 16 h cultures were washed twice with 100 mM potassium phosphate buffer, pH 7 (KP 7) and resuspended to an OD_{600} of 3.0. Assay mixtures (250 µl) contained 100 mM KP 7, 4.5 mM $MgSO_4$, 3 mM β-mercaptoethanol and 85 nM pyridoxal-5′-phosphate. Individual reactions were started by adding L-ornithine to a final concentration of 3.5 mM containing 0.025 µCi of L-[1-^{14}C]-ornithine. Assay mixtures were deposited in plastic tubes in which a CO_2 trap consisting of a 2×2.5 cm piece of filter paper wet with 125 µl of 1 M NaOH was placed so as to adhere to the top portion of the tube. In total, 50 µl aliquots of cell suspension were added to the tubes, which were sealed with rubber septa and incubated for 4 h at 30 °C. Reactions were stopped by adding 200 µl of 10 % trichloroacetic acid (TCA) and samples were re-capped and left at room temperature for 1 h. The paper CO_2 traps were mixed with 10 ml of Ultima Gold LSC cocktail (Sigma) and radioactivity determined by liquid scintillation counting. One unit (U) of activity is defined as the production of 1 nmol CO_2 min^{-1} mg $protein^{-1}$. Total cellular protein was determined as described previously [32]. Decarboxylase activities of the purified 6His-Sumo-ODC1 or 6His-Sumo-ODC2 enzymes were determined using colorimetric assays with Arg [33], Orn [34] or Lys [35] as substrates. The assay of purified enzymes was done using 25–30 µg of purified 6His-Sumo-ODC1 or 6His-Sumo-ODC2 per reaction and 1 U of activity is defined as the production of 1 nmol of decarboxylation product min^{-1} mg $protein^{-1}$. Protein concentrations were determined by the Bradford method [36].

Construction of a *odc2* transcriptional fusion with the β-glucuronidase (*gusA*) gene

odc2 is the first gene of a predicted two-gene operon but does not contain a predicted transcription start site (TSS) [37]. The PCR primers used to amplify the upstream and 5′ coding region of *odc2* are described in Table S1, and the amplified region was cloned into pTZ57R/T (Table 1). The *gusA* fusion with this gene, plasmid pBB53odc2 :: gusA, was

constructed using the PCR product of *odc2* that includes the 586 nt intergenic region between its start codon and that of the divergently transcribed *smc02984* gene, 19 and 297 nt of the *smc02984* and *odc2* coding regions, respectively. A clone containing the pTZ57R/T plasmid with the PCR product in the desired orientation was identified by digestion with appropriate restriction enzymes. The insert from the plasmid was excised with *Apa*I/*Xba*I and cloned into vector pBBMCS-53 cut likewise, transcriptionally fusing the *odc2* promoter/5′ region to the *gusA* gene. The correct transcriptional orientation of the fusion plasmid was confirmed by restriction enzyme digestion and in PCR reactions with primer p53lw (reverse primer specific for *gusA*) and the forward primer for *odc2* (Table S1; [27]). The fusion plasmid was transferred to *S. meliloti* Rm8530 by triparental mating.

β-glucuronidase (Gus) assays

Experimental cultures were grown in the desired minimal medium for 16 h at 30 °C with shaking at 200 r.p.m. Gus activity was determined by measuring the production of *p*-nitrophenol from the *p*-nitrophenyl β-D-glucuronide substrate with quantitation based on total protein [27]. One unit (U) of activity is defined as the production of 1 nmol of product min^{-1} mg $protein^{-1}$. Strain Rm8530 containing pBBMCS-53 without an insert lacked Gus activity under the growth conditions tested.

Polyamine analysis by high-performance thin-layer chromatography (HPTLC)

Dansyl (DNS) derivatives of PAs were analysed by HPTLC using modifications of previously published protocols [38, 39]. Cells from 32 h cultures were pelleted by centrifugation at 5500 ***g*** for 5 min and resuspended to an OD_{600} of 3.0 in fresh MMS. Cells from 1 ml portions of these suspensions were pelleted at 13 200 ***g***, resuspended with 0.5 ml of 5 % (w/v) TCA and stored at 4 °C for 18–24 h. Free PAs present in the TCA supernatants, obtained by centrifugation at 13 200 ***g*** for 10 min, were derivatized in 2 ml glass vials by mixing 40 µl of the supernatant, 80 µl of dansyl-chloride (DNSCl) solution (5 mg ml^{-1} in acetone) and 40 µl of supersaturated aqueous sodium carbonate. Reaction mixtures containing PA standards (1.2 µg of the PA in 40 µl of 5 % TCA) were derivatized in the same way. The capped vials were heated at 80 °C for 1 h, cooled to room temperature and quenched with 20 µl of L-proline solution (150 mg ml^{-1} in water) for 30 min at room temperature in darkness. The reaction mixtures were extracted twice with 100 µl of toluene and the combined extracts dried under a stream of N_2. Samples of DNS-PA standards and DNS-PAs from cells were resuspended with 100 and 45 µl of toluene, respectively. Then, 1 µl of DNS-PA standards or 10 µl of DNS-PAs from cells were run on Silica Gel 60 HPTLC plates (Merck) using chloroform/triethylamine (5 : 1 v/v) as the mobile phase. For the routine determination of PAs produced by *S. meliloti* cells from cultures, only the free PA fraction was analysed. PAs can exist as free molecules in the cytoplasm or as forms bound to macromolecules such as proteins, lipids or nucleic acids. These bound forms of PAs can be obtained in their free forms by strong acid hydrolysis of the TCA-precipitated macromolecules [14]. We analysed PAs bound to macromolecules as follows. Pellets of insoluble material obtained from treating cells with 5 % TCA were washed with 0.5 ml of 5 % TCA and the pellets resuspended with 0.5 ml of 6 N HCl. The suspensions were heated at 110 °C for 18–24 h in 2 ml V-Vials with teflon-lined caps (Sigma). Altogether, 20 µl of hydrolysate was combined with 40 µl each of the DNSCl and supersaturated sodium carbonate solutions described above, and derivatization and HPTLC carried out as for free PAs. Plates were visualized under UV light and images captured with a Syngene (Frederick, MD, USA) InGenius imaging system. Densitometric quantification was done using ImageJ 1.48v software. For mass spectrometric analysis, the silica gel corresponding to DNS-PA spots were scraped off the TLC plates and eluted with methanol. After passage through 0.22 µM cellulose acetate filter units (Costar), methanol was removed under a N_2 stream. The samples were reconstituted in 200 µl acetonitrile:H_2O (1 : 1; v:v) containing 0.25 % (v:v) formic acid.

Mass spectrometric analysis of dansyl-PAs

MALDI mass spectra were acquired using a Bruker 9.4T solariX XR Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer (Bremen, Germany). The samples were ionized in positive ion mode using the MALDI ion source with α-cyano-4-hydroxycinnamic acid (CHCA) matrix. Sample spots were produced by premixing 1.4 µl of sample solution with the same volume of matrix solution (saturated solution in acetonitrile:H_2O (1 : 1; v:v) containing 0.25 % (v:v) formic acid); 1 µl of this mixture was spotted onto a stainless steel target plate and allowed to air dry at ambient temperature. Spectra were measured with a transient length of 2.2 s resulting in a resolving power of 400 000 at *m/z* 400. The instrument was externally calibrated using a standard peptide mix and a 'lock-mass' calibration was used with the matrix ion with *m/z* 568.135. Collision-induced dissociation (CID) was used to generate product ions and was achieved in the hexapole collision cell using argon as the collision gas. Product ion spectra were recorded with a transient length of 1.1 s, giving a resolution of 200 000 at *m/z* 400.

RESULTS AND DISCUSSION

Identification of PAs produced by *S. meliloti* Rm8530

The work reported here uses *S. meliloti* Rm8530 as the wild-type. This strain is identical to the sequenced and well-characterized strain 1021 except that it has a functional copy of the *expR* gene, whose product is involved in quorum-sensing-based transcriptional regulation [40]. PA production by strain Rm8530 has not been reported previously. By HPTLC analysis, we found no quantitative or qualitative differences in the PAs produced by strains 1021 and Rm8530 grown under the conditions reported here (results not shown).

Selected *S. meliloti* dansyl-PA (DNS-PA) derivatives and DNS-derivatives of authentic PA standards were isolated from HPTLC plates (Fig. S1a) and analysed by MALDI high-resolution, high-mass accuracy FT-ICR mass spectrometry (MS) and product ion tandem mass spectrometry (MS/MS). The details of this analysis are described in Fig. S1. The reason for this analysis was to unambiguously identify DNS-PA spots present on our HPTLC plates, and this was particularly important for HSpd, for which no commercial standard is available, and for NSpd, which has not been reported in rhizobia.

As mentioned, PA analyses of various *S. meliloti* grown in culture showed the presence of Put, Spd, HSpd and (usually) Cad, but not Agm, spermine (Spm), NSpd or DAP. Our analysis of the PAs produced by strain Rm8530 grown in MMS (Fig. S1) shows the presence of Put, Spd, HSpd and NSpd, with the latter found only in the bound PA fraction. The presence of NSpd only in the bound fraction explains why it has not been detected previously in *S. meliloti*. Our results also indicate that Cad (or a Cad-like compound) is produced. We tentatively identified DAP in cells from PA-supplemented cultures (described later), while neither Agm nor Spm were detected under any growth condition (results not shown).

Amino acids decarboxylated by ODC1 and ODC2

To provide a biochemical basis for our assignment of the ODC1 and ODC2 proteins as a monofunctional ODC and a bifunctional Lys/ODC, respectively, we purified each as 6His-Sumo-tagged proteins and tested their ability to decarboxylate Arg, Lys and Orn. Neither protein had detectible activity with Arg as the substrate. With Orn, the 6His-Sumo-ODC1 had a specific activity of 4.1 U, but no activity with Lys as the substrate. The 6His-Sumo-ODC2 had specific activities of 8.6 and 0.9 U using Orn and Lys, respectively, as substrates. These results match our prediction of the substrates decarboxylated by each enzyme [14].

ODC2 is the major enzyme for Put synthesis in *S. meliloti*

To determine the importance of ODC1 and ODC2 in PA synthesis, we constructed single and double mutants of strain Rm8530 in which the encoding gene(s) were inactivated (Table 1). Because both the *odc1* and *odc2* genes are present in operons [37], the inactivation of either gene probably also prevents the transcription of downstream gene(s) in the operons. For *odc1*, the downstream genes encode a Put transporter (PotE; SMa0678) and a ABC transporter substrate-binding protein possibly for glutamate/aspartate (SMa0677). The products of these genes are probably not the only ones responsible for Put or glutamate/aspartate transport, since *S. meliloti* encodes an additional Put ABC transporter and three Spd/Put ABC transporters, in addition to numerous amino acid transport systems, both general and specific [14, 26]. The single downstream gene (*smc02982*) in operon with *odc2* encodes a possible *N*-acetyltransferase that we proposed might function in the production of *N*-acetylglutamate for Orn and Arg biosynthesis [26, 41]. We found, however, that a *smc02982* null mutant of *S. meliloti* 1021 was an Arg prototroph that grew normally on MMS [41].

Cultures of the wild-type and mutants were grown in MMS and their ability to decarboxylate Orn was determined. In comparison to the wild-type, the ODC activities of the Rm8530 *odc1*, *odc2* and *odc1 odc2* mutants were decreased by 14, 55 and 75 %, respectively (Fig. 2). We conclude that it is much more likely that these reductions in ODC activity result from the inactivation of *odc1* and/or *odc2* rather than of downstream genes in their operons. From these results we estimate that all but about 25 % of the total ODC activity in strain Rm8530 is due to the combined activities of the ODC1 and ODC2, with the latter enzyme accounting for about 80 % of this. The remaining ODC activity in the double mutant could result from the predicted ADC (SMa0682) being able to decarboxylate Orn in addition to or instead of Arg, or by the conversion of the ^{14}C-Orn assay substrate to ^{14}C-Arg by enzymes of the Arg biosynthesis pathway [27], with subsequent decarboxylation of the ^{14}C-Arg by the ADC.

The wild-type and mutant strains were grown in MMS under control (non-stress) or abiotic stress conditions to determine how inactivating the decarboxylases affected growth and PA production (Fig. 3). Estimations of relative changes in PA levels were made by densitometry of DNS-PA spots on HPTLC plates from at least two independent experiments. Specific growth rates (generations h^{-1}) for selected cultures are shown in Fig. 4. The growth of the mutant strains in PY rich medium was indistinguishable from that of the wild-type (results not shown).

In wild-type Rm8530 grown under control conditions, HSpd, Spd and Put account for virtually all of the DNS-PAs detected by HPTLC (Fig. 3d): relative to this total quantity set at 1.0, the proportions comprised by each of the three polyamines are 0.31, 0.56 and 0.13, respectively. During growth under the control, saline or acidic conditions, the *odc1* mutant grew similarly to the wild-type (Fig. 3a–c) and its content of HSpd, Spd and Put differed from the wild-type by less than 10 %. In contrast, the *odc2* single and *odc1 odc2* double mutants grew about 40 % slower than the wild-type or the *odc1* mutant (Fig. 4) and reached a lower cell yield under all conditions, most notably with acid stress (Fig. 3a–c). In the rhizobial plant pathogen *Agrobacterium tumefaciens* strain C58, an *odc* deletion mutant produced much less Put and Spd and grew more slowly than the wild-type in minimal medium [25]. The *A. tumefaciens* ODC and the *S. meliloti* ODC2 share over 90 % deduced amino acid sequence identity and may thus fulfill similar physiological functions.

In the wild-type under saline stress (Fig. 3d), HSpd was undetectible and Put decreased by >90 %, but Spd levels were maintained at a high level similar to that seen under control conditions. HSpd levels decrease in salt-stressed

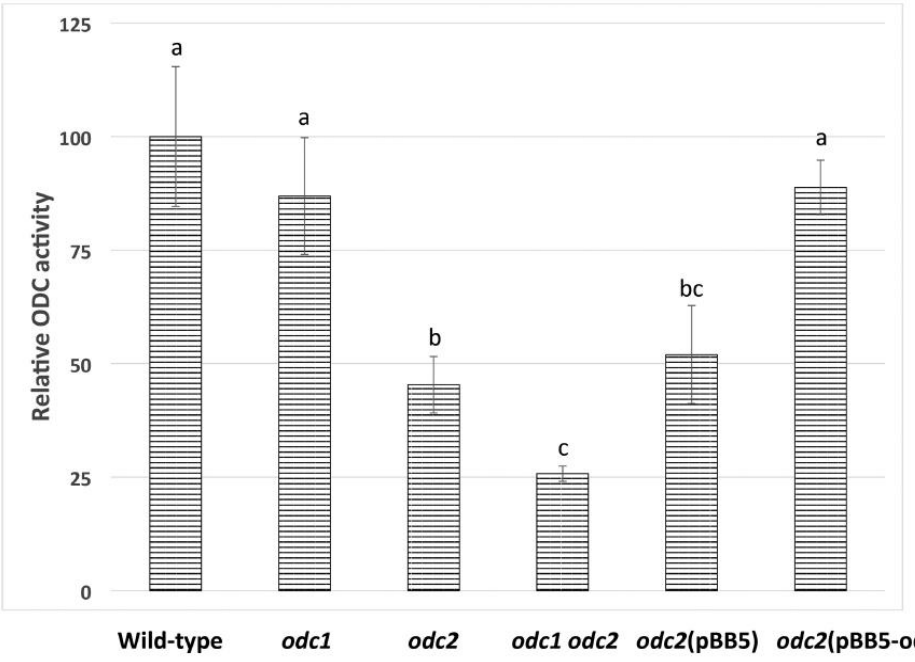

Fig. 2. Specific activities of Orn decarboxylation by *S. meliloti* strains grown in MMS, normalized to that of the Rm8530 wild-type (100 %=3968 U). Values are the mean±SD for two independent experiments. Values for columns marked with the same letter are not statistically different according to Student's *t*-test.

Sinorhizobium fredii P220 (a relatively salt- and acid-resistant strain), where it was proposed that having less of this polycation offsets the increase in positive charges caused by the rise in cytosolic K^+ that occurs under these conditions [18]. With acidic stress, wild-type Rm8530 had a nearly fourfold decrease in Put, while HSpd and Spd levels remainded constant. In *S. fredii* P220 HSpd levels increase twofold at pH 4 as compared to pH 9.5, which is an acidic stress much more drastic than the pH 5.5 versus pH 6.8 stress that we imposed on *S. meliloti* in our experiments. Under acidic conditions, HSpd may provide cytosolic buffering or protect macromolecules from acid denaturation [18].

PA levels in the *odc1* mutant differed by no more than 10 % from the wild-type under all of the growth conditions tested. The *odc2* mutant grown under control conditions lacked detectable Put and produced 9 and 18 % the wild-type levels of HSpd and Spd, respectively. It also contained some apparent NSpd in the free fraction that accounted for 4.3 % of the total PAs. The PA profile of the *odc1 odc2* double mutant from control cultures was similar to that of the *odc2* single mutant (including the presence of free NSpd), except that Put was present at 11 % of the wild-type level. The reduction in growth caused by the inactivation of the *S. meliloti odc2* is similar to what occurs in *R. leguminosarum* and *A. tumefaciens* when Put synthesis is lowered by treatment with the ODC inhibitor dimethylfluoroornithine or by inactivation of the *odc* gene, respectively [23, 25]. In both the *odc2* and double mutants, the levels of Put, Spd and HSpd were also markedly lower than in the wild-type during growth under salt or acid stress (Fig. 3d).

Chemical complementation restores growth and PA levels in the *odc2* mutant

Testing the ability of an exogenous PA to restore the normal phenotype of a PA mutant is called chemical complementation [9, 11, 23, 24]. The effect of chemical complementation on the specific growth rate (μ, or generations h^{-1}; Fig. 4) and PA content (Fig. 5) of the Rm8530 wild-type and selected PA mutants was determined under control, salt stress and acid stress conditions. For these experiments, we used exogenous Put and Spd for chemical complementation since these PAs result directly or indirectly from Orn decarboxylation (Fig. 1). HSpd is derived directly from Put but was not tested because it is not commercially available. NSpd was used for chemical complementation since its synthesis does not require Put (Fig. 1).

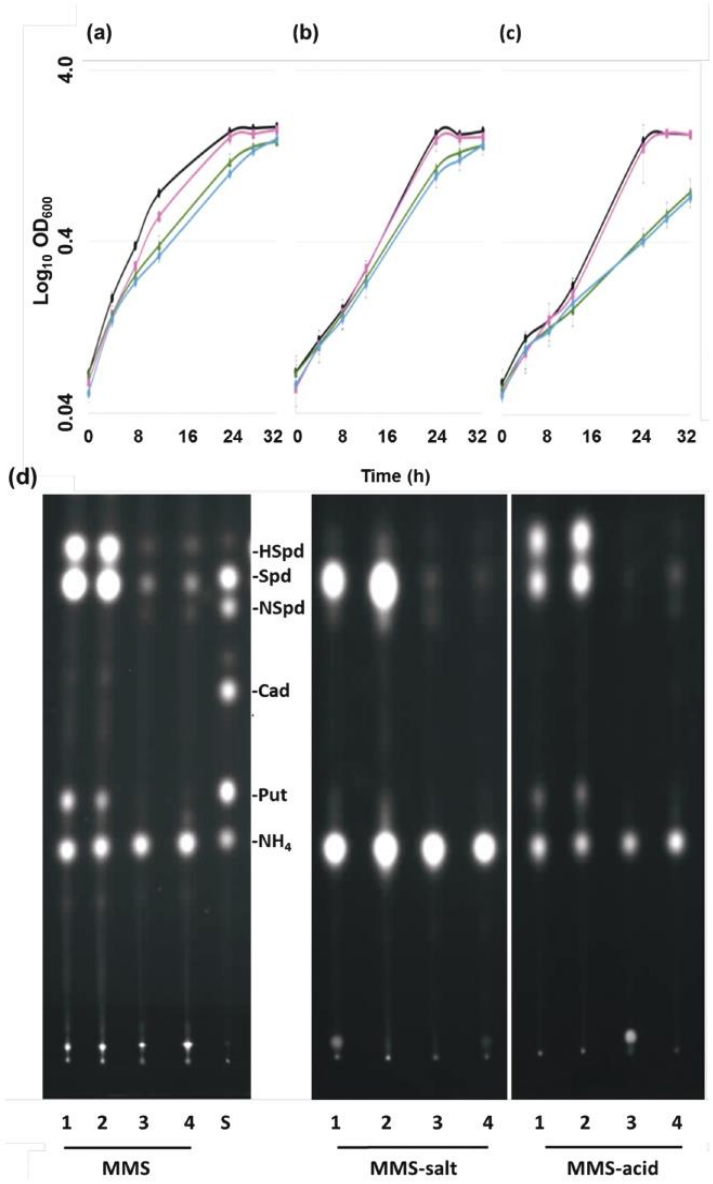

Fig. 3. Growth and PA content of *S. meliloti* cultured under non-stress and stress conditions. (a–c) represent culture growth under control, salt stress and acidic stress conditions, respectively. Strains and line colours: Rm8530 wild-type, black; 8530 *odc1*, pink; 8530 *odc2*, green; 8530 *odc1 odc2*, blue. (d) shows HPTLC detection of dansyl-PAs from 32 h cultures. Lane S contains dansyl-PA standards with their identities shown at the right side of the first plate image. *S. meliloti* dansyl-PA samples are: lane 1, Rm8530; lane 2, 8530 *odc1*; lane 3, 8530 *odc2*; lane 4, 8530 *odc1 odc2*.

In control (non-stressed) cultures without added PAs, μ values of the *odc1*, *odc2* and *odc1 odc2* mutants were 93, 59 and 60 % that of the wild-type (Fig. 4a). The specific growth rate of the *odc1* mutant grown under stress or non-stress conditions with or without exogenous PAs differed from the wild-type by no more than 13 %, in contrast to the much

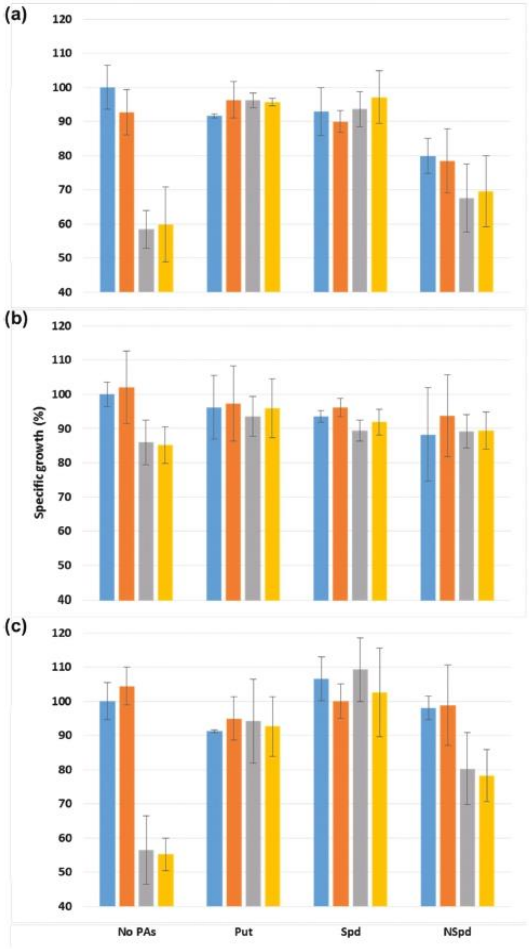

Fig. 4. Specific growth rates (μ, generations h^{-1}) of selected *S. meliloti* strains grown in MMS with or without chemical complementation with exogenous PAs. (a) Control conditions; (b) MMS-salt; (c) MMS-acid. PA added to the cultures is indicated at the bottom of the figure. Bar colours represent: Rm8530 wild-type, blue; 8530 *odc1*, orange; 8530 *odc2*, grey; 8530 *odc1 odc2*, yellow. Values are normalized to μ values of the wild-type grown under the three conditions in media lacking added PAs, where 100 % corresponds to μ values of 0.179, 0.141 and 0.130 for the MMS, MMS-salt and MMS-acid cultures. Results are the mean±SD for two independent experiments.

more pronounced growth defects found in the *odc2* and *odc1 odc2* mutants. As mentioned, the levels of Put, HSpd and Spd in the *odc1* mutant are comparable to those of the wild-type, while they are similarly and drastically reduced to low levels in the *odc2* and double mutants (Fig. 3d). In cultures without added PAs, growth under salt and acidic stress reduced the μ of Rm8530 by 21 and 27 %, respectively, in comparison to non-stress conditions (Fig. 4). Under salt stress conditions, the μ values of the mutants were less affected relative to the wild-type grown under the same condition, with the *odc1*, *odc2* and double mutants having 102, 86 and 85 % wild-type growth rates (Fig. 4b). This may occur because *S. meliloti* responds to salt stress by lowering its total PA content (Fig. 3d), and so the *odc2* and double mutants, with their very low PA levels, are less affected for growth under saline conditions than under control conditions. Under acid stress, the *odc1*, *odc2* and double mutants had μ values of 105, 57 and 55 % of wild-type (Fig. 4c). Thus, under this stress condition, the lack of ODC1 activity has essentially no effect on growth, while the mutants lacking ODC2 activity have growth reductions similar to that found under control conditions.

For wild-type Rm8530 grown under control (non-stress) conditions (Fig. 4a), exogenous Put or Spd caused a reduction in μ of 8–9 % and NSpd caused a 21 % decrease. Lesser decreases in wild-type μ values were caused by the PAs under salt stress (reductions of 4, 6 and 12 % for Put, Spd and NSpd, respectively). Under acid stress, wild-type μ values decreased 7 % with Put, increased 1.1-fold with Spd and were unchanged with NSpd. For the *odc2* and double mutant grown under stress or non-stress conditions, Put supplementation restored μ to 94–96 % to that of the wild-type. When these mutants were grown under control or salt stress conditions, exogenous Spd restored growth rates to 89–96 % to that of the wild-type, while under acid stress it allowed growth at slightly higher than wild-type μ values (Fig. 4). When grown in NSpd-supplemented cultures under non-stress conditions, the growth of the *odc2* and double mutants were restored to only 66–69 % to that of the wild-type, respectively. Under salt and acid stress, these values ranged from 79–89 % to the wild-type. Thus, exogenous NSpd was not as effective as Put or Spd in restoring the growth of *odc2* and double mutants under any growth condition. It is interesting to note that the growth restoration caused with NSpd was greater under stress than non-stress conditions.

Under control conditions, Rm8530 cells from cultures grown with 1 mM Put (Fig. 5a) had 77 % less Put, 30 % less Spd and 1.9-fold more HSpd relative to cells grown without added Put (Fig. 3d and results not shown). Cells from the Put-supplemented cultures also contained a trace of DAP, which accounted for 1 % of the total PAs present (Fig. 5a). The decrease in Put might result from its use in HSpd synthesis. DAP is not derived from Put and it is not known whether Put can modulate the production of PAs in the L-aspartate β-semialdehyde (L-Asp β-SA) branch of the synthesis pathway (Fig. 1).

Supplementation of Rm8530 control cultures with Spd caused a modest (13 %) decrease in intracellular Spd concentration, a 1.6-fold increase in Put and no change in HSpd, but caused the appearance of detectible DAP (Figs 3d, 5b and results not shown). As described later, the drastic reduction in *odc2* transcription observed in cells

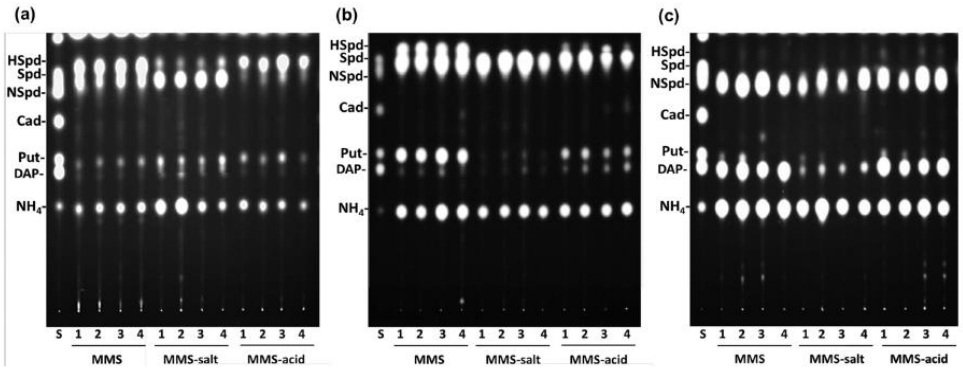

Fig. 5. Effect of chemical complementation with exogenous PAs on PA production by *S. meliloti* strains. HPTLC detection of dansyl-PAs from 32 h cultures. Lane S contains dansyl-PA standards with their identities shown at the left side of the plates. (a) MMS plus 1 mM Put; (b) MMS plus 1 mM Spd; (c) MMS plus 1 mM NSpd. Lane assignments for all plates are as follows: lane 1, Rm8530 wild-type; lane 2, 8530 *odc1*; lane 3, 8530 *odc2*; lane 4, 8530 *odc1 odc2*.

from Spd-supplemented cultures suggests that the nearly unchanged level of intracellular Spd could result from its reduced synthesis by ODC2 being offset by the uptake of exogenous Spd. Whether the increase in Put is derived from the retroconversion of Spd to Put, as occurs in *A. tumefaciens* [11], is unknown. In Spd-supplemented MMS-salt cultures, HSpd was undetectable while Put and DAP levels were greatly decreased in comparison to their levels in the control cultures. Under these conditions, Spd was the only PA present at high levels. Cultures grown under acidic conditions with added Spd produced significantly less HSpd and Put than non-stressed cultures, but had the same or slightly higher levels of DAP. For all of the strains grown under all conditions, the addition of NSpd to the medium resulted in a high level of its accumulation, an apparent total absence of Spd and at most trace levels of HSpd and Put, and remarkably high amounts of DAP (Fig. 5c). In *A. tumefaciens*, cells grown in the presence of NSpd (which is not produced by this organism) also produce little intracellular Spd [25]. In *S. meliloti*, the intriguing possibility exists that NSpd has regulatory effects on the production on PAs derived from Put.

Genetic complementation restores growth and PA levels in the *odc2* mutant

To confirm that the inactivation of the *odc2* gene was responsible for the altered phenotype of the *odc2* mutant, we introduced the *odc2* gene into the mutant on plasmid pBB5-*odc2* (Table 1). The resulting transconjugant, 8530 *odc2* (pBB5-*odc2*), had its Orn decarboxylating activity restored to nearly that of the wild-type, while the activity in the mutant containing the cloning vector alone (strain 8530 *odc2*(pBB5)) was very similar to that of the uncomplemented mutant (Fig. 2). Strain 8530 *odc2*(pBB5-*odc2*) also grew similarly to the wild-type under stress and non-stress growth conditions, and its ability to produce PAs was restored to approximately wild-type levels (Fig. 6). These results are consistent with the inactivation of the *odc2* gene being the cause of the growth and PA production phenotypes of the *odc2* mutant. The *odc2* mutant also produced some apparent NSpd in the free PA fraction (Fig. 6d). In comparison to the uncomplemented mutant, strain 8530 *odc2*(pBB5) had a slower growth rate under all conditions, perhaps due to the metabolic burden of plasmid maintenance. As expected, strain 8530 *odc2*(pBB5) produced low levels of PAs similar to the mutant without the plasmid (Fig. 6).

Although the introduction of genes for metabolic enzymes cloned on plasmid pBB5 into *S. meliloti* can increase the gene products' activity several-fold, presumably due to increased copy number [27], we did not find increased ODC activity (Fig. 2) or overproduction of PAs (Fig. 6d) in strain 8530 *odc2*(pBB5-*odc2*).

Transcriptional expression of *odc2* under different growth conditions

The expression of β-glucuronidase (*gusA*) transcriptional fusions to the *odc1* and *odc2* genes was determined in strain Rm8530 grown under different growth conditions. The *odc1* gene is part of a predicted four-gene operon and lacks a transcriptional start site (TSS) [37]: we found that a *odc1::gusA* fusion produced a low level of Gus activity (50–115 nmol min^{-1} mg $protein^{-1}$). *In vivo*, *odc1* transcription may be linked to that of the upstream gene (*smc0682*), which is not part of the fusion construct.

Under the growth conditions tested, the GUS specific activity from the *odc2::gusA* fusion changed over a sevenfold range, from about 400 to 2900 nmol min^{-1} mg $protein^{-1}$ (Fig. 7). Although *odc2* also lacks a recognizable TSS, it is

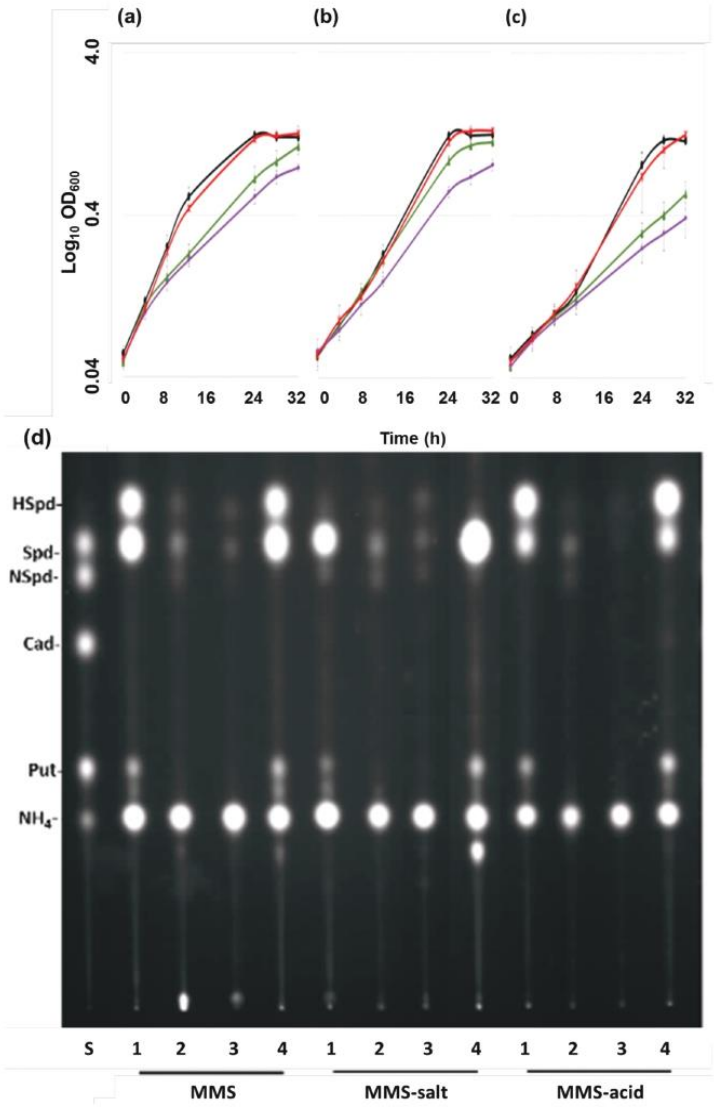

Fig. 6. Growth and PA content of the genetically complemented 8530 *odc2* mutant. (a–c) show culture growth under control, salt stress and acidic stress conditions, respectively. Strains and line colours: Rm8530 wild-type, black; 8530 *odc2*, green; 8530 *odc2* (pBB5-*odc2*), red; 8530 *odc2*(pBB5), purple. (d) shows HPTLC detection of dansyl-PAs from 32 h cultures. Lane S contains dansyl-PA standards with their identities shown at the left side of the plate. *S. meliloti* dansyl-PA samples are: lane 1, Rm8530; lane 2, 8530 *odc2*; lane 3, 8530 *odc2*(pBB5); lane 4, 8530 *odc2*(pBB5-*odc2*).

the first gene in an apparent two-gene operon [37] and the results described below suggest that *odc2* expression is modulated in response to growth conditions.

In comparison to control (non-stress) conditions, *odc2* transcription decreased 69 % under salt stress (Fig. 7), consistent with the 94 % reduction in Put in these cells (Fig. 3d) and results not shown). The decreased Put content in salt-stressed cells is not explained by its conversion to HSpd and/or Spd, since the combined quantity of these PAs was nearly identical in the control and salt-stressed cells. Decreased expression of *odc2* was also reported in a transcriptomic study of *S. meliloti* 1021 grown under salt stress

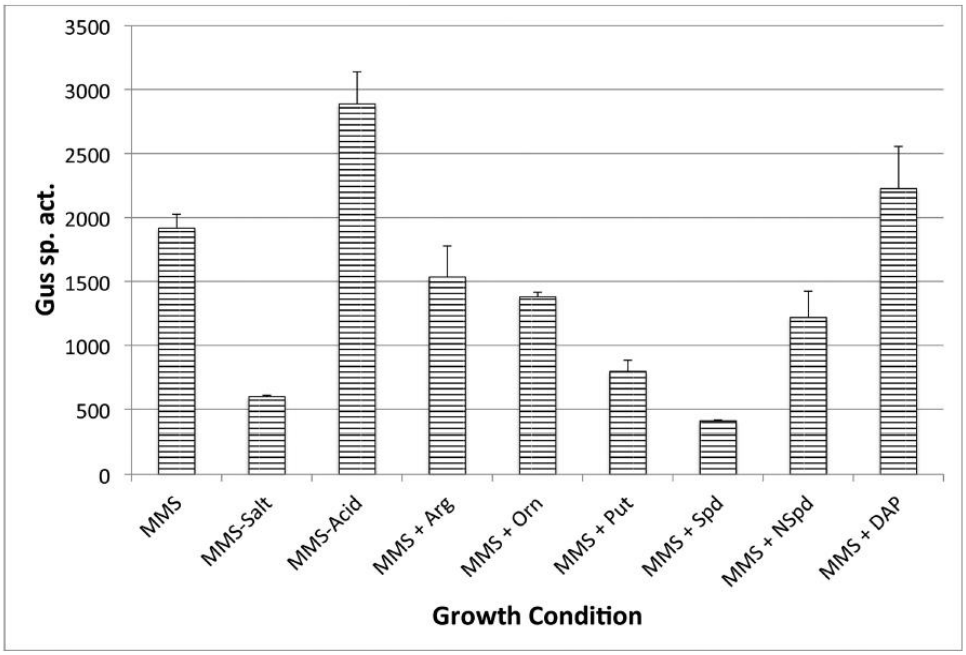

Fig. 7. β-glucuronidase (Gus) activities produced by *S. meliloti* Rm8530 containing the *odc2 :: gusA* transcriptional fusion plasmid. Cells were grown in the indicated media for 16 h. Values are the mean±SD for two independent experiments, each with two technical replicates of two biological replicates. 1 U=nmol product min^{-1} mg $protein^{-1}$.

[42]. Acidic stress increased *odc2* transcription 1.5-fold, which does not correlate with the 77 % decrease in Put levels seen during growth at low pH (Fig. 3d).

Cells grown in cultures supplemented with the exogenous Put precursor amino acids Arg or Orn expressed *odc2* at a level 20 and 28 % less than in cells from unsupplemented cultures. Assigning these effects solely to the exogenous amino acid added to the cultures is complicated by the ability of *S. meliloti* to convert Arg to Orn using arginase and to metabolize Orn to Arg by activities of the Arg synthesis pathway [14]. We can tentatively conclude that Orn, the major substrate for ODC2, does not induce *odc2* expression.

To determine the effect of exogenous PAs on *odc2* transcription, we used Put and Spd, which result directly and indirectly from Orn decarboxylation, respectively, and DAP and NSpd, which are not derived from Put (Fig. 1). Exogenous Put, Spd and NSpd inhibited *odc2* transcription by 58, 79 and 36 % respectively, while DAP resulted in a small increase in its transcription (Fig. 7). Thus, the PA products resulting from Orn decarboxylation inhibited *odc2* transcription to a greater degree than the PAs from the L-Asp β-SA branch of the pathway.

In summary, we have shown that Put and/or PAs derived from it are required for the normal growth of *S. meliloti* Rm8530. ODC2 (SMc02983) is a bifunctional Lys/Orn decarboxylase responsible for synthesizing the majority of Put produced by strain Rm8530, and changes in *odc2* transcription observed under some growth conditions are consistent with observed changes in PA levels. The *S. meliloti* ODC1 (SMa0680) in a monofunctional ODC that contributes a minor portion of the ODC activity in Rm8530.

The results presented here provide a basis for further experiments aimed at deciphering the enzymology and regulation of PA metabolism in *S. meliloti*, which provides an attractive model system due to its extended PA biosynthetic capabilities [14]. We are currently addressing some of these questions, along with determining the physiological roles of specific PAs in free-living and symbiotically associated *S. meliloti*.

Funding information
Work at the UNAM was supported by DGAPA-PAPIIT Grants IN210114 and IN206317 to M. F. D. The York Centre of Excellence in Mass Spectrometry was created thanks to a major capital investment through Science City York, supported by Yorkshire Forward with funds from the Northern Way Initiative, and subsequent support from EPSRC (EP/K039660/1; EP/M028127/1).

Acknowledgements
Victor A. Becerra-Rivera is a doctoral student from Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de

México (UNAM) and received fellowship 574042 from CONACYT. We thank María J. Soto (Departamento de Microbiología del Suelo y Sistemas Simbióticos de la Estación Experimental del Zaidín, CSIC, Granada, Spain) for providing *S. meliloti* strain Rm8530 and Lourdes Girard (CCG-UNAM) for providing the p53lw primer and the pBBRMCre plasmid.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Edited by: I. J. Oresnik and G. H. Thomas

MICROBIOLOGY

RESEARCH ARTICLE

Becerra-Rivera *et al.*, *Microbiology*

DOI 10.1099/mic.0.000886

Polyamines produced by *Sinorhizobium meliloti* Rm8530 contribute to symbiotically relevant phenotypes *ex planta* and to nodulation efficiency on alfalfa

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Abstract

In nitrogen-fixing rhizobia, emerging evidence shows significant roles for polyamines in growth and abiotic stress resistance. In this work we show that a polyamine-deficient ornithine decarboxylase null mutant (*odc2*) derived from *Sinorhizobium meliloti* Rm8530 had significant phenotypic differences from the wild-type, including greatly reduced production of exopolysaccharides (EPS; ostensibly both succinoglycan and galactoglucan), increased sensitivity to oxidative stress and decreased swimming motility. The introduction of the *odc2* gene borne on a plasmid into the *odc2* mutant restored wild-type phenotypes for EPS production, growth under oxidative stress and swimming. The production of calcofluor-binding EPS (succinoglycan) by the *odc2* mutant was also completely or mostly restored in the presence of exogenous spermidine (Spd), norspermidine (NSpd) or spermine (Spm). The *odc2* mutant formed about 25% more biofilm than the wild-type, and its ability to form biofilm was significantly inhibited by exogenous Spd, NSpd or Spm. The *odc2* mutant formed a less efficient symbiosis with alfalfa, resulting in plants with significantly less biomass and height, more nodules but less nodule biomass, and 25% less nitrogen-fixing activity. Exogenously supplied Put was not able to revert these phenotypes and caused a similar increase in plant height and dry weight in uninoculated plants and in those inoculated with the wild-type or *odc2* mutant. We discuss ways in which polyamines might affect the phenotypes of the *odc2* mutant.

INTRODUCTION

Polyamines (PAs) are low-molecular-weight aliphatic polycations that have important and often essential functions in prokaryotes and eukaryotes. In bacteria, PAs are in some cases essential for growth and also have important roles in translation, exopolysaccharide (EPS) synthesis, biofilm formation, stress resistance, motility, plant growth promotion and the virulence of plant and animals pathogens [1–4]. The diamine putrescine (Put) is produced directly by the decarboxylation of ornithine or in a two-step pathway that begins with the decarboxylation of arginine. Put is a precursor of several other PAs, including the triamines spermidine (Spd) and homospermidine (HSpd) [5].

Symbiotic biological nitrogen fixation is an economically and ecologically important process in which certain α- and β-proteobacteria reduce atmospheric nitrogen to ammonia within root nodules that they induce on legumes. Among the microsymbiont's traits that are required to establish symbiosis with the host are the ability to move from the bulk soil to the rhizosphere, the synthesis of nodulation factors and EPS and the formation of biofilms on the root. Rhizobia also need to withstand oxidative stress and perhaps other abiotic stresses during the nodulation process (for reviews, see [6–11]).

Several studies have provided evidence for the physiological importance of PAs in free-living and symbiotically associated rhizobia (reviewed in [7, 12]). For instance, a HSpd synthase (Hss) null mutant of *Rhizobium tropici* CIAT899 had a lower

Received 19 August 2019; Accepted 23 December 2019; Published 14 January 2020

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Keywords: ornithine decarboxylase; putrescine; spermidine; homospermidine; rhizobia–legume symbiosis.

Abbreviations: μ, specific growth rate; AR, acetylene reduction; CV, crystal violet; DAP, 1,3-diaminopropane; EPS, exopolysaccharide; EPS I, succinoglycan; EPS II, galactoglucan; HPTLC, high performance thin layer chromatography; HSpd, homospermidine; Hss, homospermidine synthase; MOPS, 3-(N-morpholino)propanesulfonic acid; NSpd, norspermidine; OD, optical density; Odc, ornithine decarboxylase; PA, polyamine; Put, putrescine; Spd, spermidine; Spm, spermine.

Supplementary material is available with the online version of this article.

tolerance to salt stress in cultures but was symbiotically similar to the wild-type in combination with bean, even when plants were subjected to salt stress [13]. A *Rhizobium etli* CNPAF512 *hss* null mutant was unable to swarm and had slightly slower growth as compared to the wild-type and formed nodules with normal nitrogen-fixing activity on bean [14]. In the rhizobial plant pathogen *Agrobacterium tumefaciens* C58, the level of PAs modulates the switch between planktonic growth and biofilm formation, and Spd or 1,3-diaminopropane (DAP) are essential for growth [15, 16].

We showed previously that a *Sinorhizobium meliloti* Rm8530 ornithine decarboxylyase mutant (strain *odc2*) produced very low levels of Put, HSpd and Spd and had slower growth than the wild-type, especially under acidic or salt stress conditions [17]. Work with different bacterial model organisms has shown that motility, stress resistance and EPS production are affected by changes in intracellular PA levels [2, 7]. In this work, we extend our characterization of the *S. meliloti odc2* mutant and show that it has reduced swimming motility, produces less EPS but makes more biofilm, is more sensitive to oxidative stress and has lowered symbiotic efficiency with alfalfa. The possible role of PAs in determining these phenotypes is discussed.

METHODS

Bacterial strains, plasmids and culture growth conditions

The bacterial strains and plasmids are listed in Table 1. Wild-type strain *S. meliloti* Rm8530 is identical to strain 1021, except for having a functional copy of the transcriptional regulator gene *expR*, which is essential for quorum sensing and allows the production of galactoglucan (EPS II) in addition to succinoglycan (EPS I). PY complex medium and MMS-NH4 minimal medium, with succinate and NH_4Cl as carbon and nitrogen sources, respectively, were described previously [18]. Oxidative stress was induced by growing cultures in MMS-NH4 containing 0.33 mM H_2O_2. PA stocks for use in chemical complementation were prepared at 0.2 M, adjusted to pH 6.8 and filter sterilized. For the growth of *S. meliloti* strains in MMS-NH4, cells from 3-day PY plates with appropriate antibiotics were used to inoculate 3 ml of liquid PY containing the same antibiotics and incubated at 200 r.p.m., 30 °C. After 24 h, 1 ml of these cultures was used to inoculate 50 ml of PY containing one-half of the normal concentration of antibiotics, in 125 ml baffled flasks. These cultures were incubated as above for 24 h and harvested by centrifugation at 5500 ***g*** for 5 min, and the cells washed twice in MMS-NH4 and resuspended to an optical density (OD) at 600 nm (OD_{600}) of approximately 1.5 in MMS-NH4. These suspensions were used to inoculate the desired minimal medium without antibiotics to an initial OD_{600} of 0.05. For culture growth (OD_{600}) determinations, 50 ml cultures in MMS-NH4 were grown in 125 ml baffled flasks with agitation at 200 r.p.m. at 30°C. Specific growth rates (μ, generations h^{-1}) and total protein content were determined as described previously [17]. When required, media contained antibiotics at the following concentrations (μg ml^{-1}): gentamicin (Gm), 15; spectinomycin (Sp), 100; streptomycin (Sm), 200.

EPS quantification

The production of succinoglycan (EPS I) by *S. meliloti* was determined by a semiquantitative assay using calcofluor [19]. Overnight cultures in 3 ml PY medium with antibiotics were washed twice in MMS-NH4 and diluted to an OD_{600} of 0.2. Five microlitres of this dilution was pipetted onto MMS-NH4

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
S. meliloti strains		
Rm8530	*S. meliloti* 1021 *expR*$^+$, Sm^r	M. Soto, Estación Experimental del Zaidín, CSIC, Granada, Spain
odc1	Rm8530 *sma0680*::*loxP* Sp; *odc1* null mutant, Sm^r Sp^r	[17]
odc2	Rm8530 *smc02983*::*loxP* Sp; *odc2* null mutant, Sm^r Sp^r	[17]
odc1 odc2	Rm8530 *sma0680*::*loxP smc02983*::*loxP* Sp; *odc1 odc2* null double mutant, Sm^r Sp^r	[17]
odc2(pBB5)	Rm8530 *smc02983*::*loxP* Sp *odc2* null mutant containing plasmid pBB5, Sm^r Sp^r Gm^r	[17]
odc2(pBB5-odc2)	Rm8530 *smc02983*::*loxP* Sp *odc2* null mutant complemented *in trans* with the *odc2* gene, Sm^r Sp^r Gm^r	[17]
Plasmids		
pBB5	Broad-host-range vector pBBR1MCS-5, Gm^r	[55]
pBB5-odc2	pBBR1MCS-5 containing *smc02983* with native promoter and terminator regions, Gm^r	[17]

plates with or without exogenous PAs (1 mM), and containing 0.02% Calcofluor White M2R (Sigma-Aldrich, St. Louis, MO, USA). After 3 days of incubation at 30 °C, EPS production was visualized under UV light. To quantitate total EPS production in liquid cultures, the strains were grown in MMS-NH4 to an OD_{600} of ~1 and centrifuged for 10 min at 12500 ***g***. The supernatant was carefully decanted, mixed with three volumes of cold 95% (v/v) ethanol and stored at −20 °C overnight. The precipitated EPS was pelleted by centrifugation at 15000 ***g*** for 10 min, resuspended in 0.75 ml of 100 mM KCl/1 mM EDTA and dialysed against 1 mM EDTA for 24 h, followed by exhustive dialysis versus water [20]. The samples were dried by lyophylization and weighed.

Motility assays

Swimming assays were performed in semisolid Bromfield medium [0.04% tryptone, 0.01% yeast extract, 0.01%$CaCl_2 \cdot 2H_2O$, 0.3% Difco Noble Agar (Becton, Dickison and Co., Sparks, MD, USA)]. Growth from individual colonies of *S. meliloti* strains grown on PY plates was transferred to a Bromfield plate with a toothpick. After 3 days of incubation at 30 °C, areas corresponding to cellular displacement were calculated from the diameters of the growth zones [21].

Biofilm assays

Biofilm formation by cultures grown in polystyrene 96-well microplates (Costar, Corning, NY, USA) was determined by crystal violet (CV) staining essentially as described by O'Toole and Kolter [22]. Overnight cultures of *S. meliloti* strains were grown in 3 ml PY medium with antibiotics and cells were washed twice in MMS-NH4 and diluted to an OD_{600} of 0.2. One hundred and eighty microlitres of suspension was added per well of the microplate, which was covered with sterile breathable sealing film (Axygen, Corning, NY, USA) and incubated for 72 h at 40 r.p.m. 30 °C. Bacterial growth was quantified (OD_{595}) before removal of the planktonic cells by gentle aspiration. Biofilms were stained for 15 min with 180 µl of 0.1% CV per well. Each CV-stained well was rinsed three times with water and air-dried. The CV was resolubilized by adding 180 µl of 95% (v/v) ethanol to each well, mixing by repeated aspiration and reading the OD at 595 nm (3550 Microplate Reader, Bio-Rad, Hercules, CA, USA).

Medicago sativa–S. meliloti symbiosis assays

M. sativa (var. Cuf101, Rancho Los Molinos, Tepoztlán, Morelos, Mexico) seeds were washed three times with distilled water and 95% (v/v) ethanol, followed by 2 h in 50% (v/v) commercial bleach with gentle mixing on a rocker platform. Seeds were rinsed five times in sterile water and placed onto 1% water–agar plates for 3 days at 30 °C in darkness. The germinated seedlings were transferred to pots with sterile vermiculite and inoculated with 150 µL of an overnight culture of *S. meliloti* grown in PY. After 2 days of adaptation, plants were watered every third day with a nitrogen-free nutrient solution (1 mM $CaCl_2 \cdot 2H_2O$, 0.84 mM $Na_2HPO_4 \cdot 7H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, 0.73 mM KH_2PO_4, 0.02 mM $FeCl_3 \cdot 6H_2O$, 1.24 µM $MgSO_4$, 1.98 µM H_3BO_3, 0.49 µM $ZnSO_4 \cdot 7H_2O$, 1.98 µM $CuSO_4 \cdot 5H_2O$, 0.1 µM $CoCl_2 \cdot 6H_2O$, 0.1 µM $Na_2MoO_4 \cdot 2H_2O$) and on other days with water. For Put supplementation, nutrient solution containing 1 mM Put was used for watering every third day. Plants were grown in growth chambers under controlled environmental conditions (25–28 °C, 16 h photoperiod). Forty-five days after inoculation, the plants were measured and harvested. Nodules and leaves were frozen at 80 °C until PA analysis. Nitrogenase activity was determined in nodulated roots using the acetylene reduction (AR) assay as described by Hardy *et al.* [23]. Nodules and plant above-ground parts were dried for 3 days at 65 °C before dry weight determination.

PA analysis by high-performance thin-layer chromatography (HPTLC)

Free PAs in cells from 28 h cultures were analysed as described by Becerra-Rivera *et al.* [17]. To determine the PAs in leaves and nodules, 200 mg of extracts of either tissue was prepared in 0.6 ml of 5% (v/v) cold perchloric acid and incubated for 24 h at 4 °C. The mixtures were centrifuged at 3000 ***g*** for 5 min at 4 °C. Derivatization was performed by mixing 0.2 ml of the supernatant with 0.4 ml of dansyl chloride solution (10 mg ml^{-1} in acetone) and 0.2 ml of supersaturated aqueous sodium carbonate. Reaction mixtures containing PA standards (1.2 µg of the polyamine in 40 µl of 5% trichloroacetic acid) were derivatized in the same way. Reactions containing tissue samples or standards were incubated in darkness at room temperature overnight. Excess dansyl reagent was removed by adding 0.1 ml of L-proline solution (100 mg ml^{-1}) followed by incubation for 30 min. Dansyl–PAs were extracted with 0.5 ml toluene and dried under a stream of N_2 [24]. The dansyl–PAs were resuspended in 100 µl of toluene and 1 µl of dansyl–PA standards or 10 µl of dansyl–PAs from leaves and nodules were run on Silica Gel 60 HPTLC plates (Merck, Darmstadt, Germany) using chloroform/triethylamine (5:1 v/v) as the mobile phase. Plates were visualized under UV light and images were captured with a Syngene imaging system (Syngene, Frederick, MD, USA).

RESULTS

Polyamines affect EPS production in *S. meliloti*

S. meliloti strains produce succinoglycan (EPS I), which when bound to the laundry brightener calcofluor fluoresces under UV light. Agar plates containing calcofluor thus provide a widely accepted semiquantitative assay for EPS I production [19, 25]. Our assays were performed on agar plates prepared with MMS-NH4 with a pH of 6.8 prior to autoclaving. The low fluorescence produced by the *odc2* mutant in the calcofluor staining assay indicates that it produces less EPS I than the wild-type (Fig. 1a). However, the intensity of calcofluor staining is markedly influenced by the pH of the growth medium. Hawkins *et al.* [26] reported that calcoflour assays with *S. meliloti* 1021 spotted on minimal medium (mannitol–ammonium nitrate) buffered at pH 6.5 and 6.0 resulted in bright and dim fluorescence, respectively. Our assays with the wild-type and the *odc2* mutant gave indistinguishable results

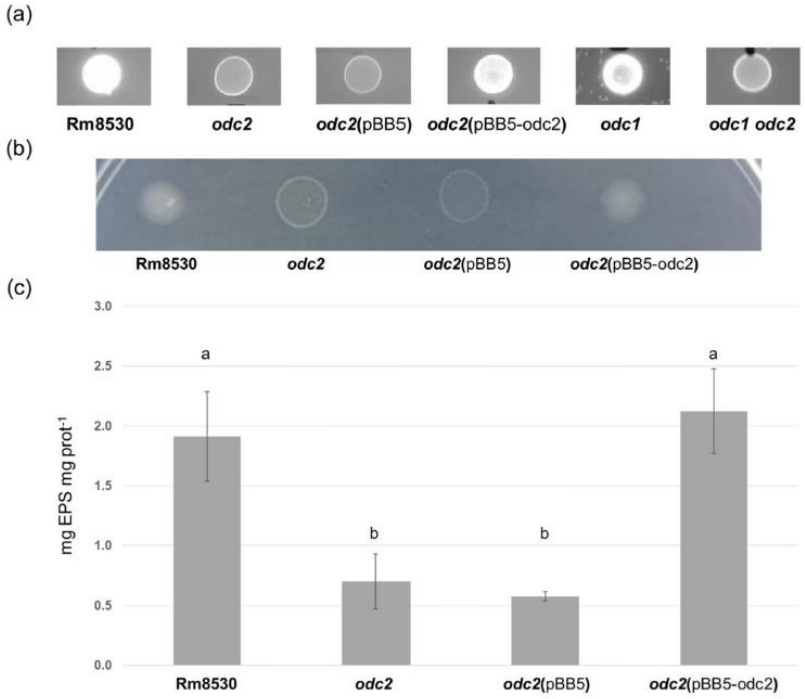

Fig. 1. Polyamines modify EPS synthesis in *S. meliloti*. (a) Assay for EPS I production on calcofluor-containing minimal medium plates photographed under UV light. The tested strains were Rm8530 wild-type, the *odc2* mutant, the *odc2* strain containing empty vector [odc2(pBB5)], the *odc2* mutant complemented *in trans* with the *odc2* gene [*odc2*(pBB5-odc2)], and the *odc1* single mutant and *odc1 odc2* double mutant. High fluorescence (brightness) is indicative of high EPS I production. (b) Relative mucoidy of growth of Rm8530 strains on minimal medium. The *odc2* mutant and the *odc2* mutant with empty vector pBB5 have non-mucoid phenotypes, indicating low EPS II production. (c) Total EPS produced in minimal medium liquid cultures (quantitated after purification from supernatants) by strains Rm8530 wild-type, *odc2* mutant, *odc2* mutant with empty vector [*odc2*(pBB5)] and *odc2* mutant complemented *in trans* with the *odc2* gene [*odc2*(pBB5-odc2)]. Values are the mean±SD for three independent experiments. The columns marked with the same letter are not statistically different according to the LSD Fisher test.

on calcoflour plates prepared with unbuffered or buffered (50 mM MOPS) MMS-NH4 adjusted to pH 6.8 prior to autoclaving. (V. A. Becerra-Rivera, unpublished results).

The *odc1 odc2* double mutant also had a dim fluorescence phenotype, while the *odc1* mutant and wild-type Rm8530 were highly fluorescent (Fig. 1a). The *odc1* mutant was previously shown to be very much like the wild-type in terms of PA production and growth phenotypes [17]. A bright calcofluor phenotype resulted when the *odc2* mutant was complemented *in trans* with a plasmid containing the *odc2* gene [strain *odc2*(pBB5-odc2)], but not when the empty vector was introduced (Fig. 1a).

We evaluated the ability of exogenously supplied PAs (1 mM) to chemically complement the EPS I deficiency of the *odc2* mutant. For these assays, we used Spd, which is synthesized from Put; norspermidine (NSpd), which does not require Put as a precursor; and Spm, which is not synthesized by *S. meliloti* [7]. The calcofluor plate assays showed that the triamines Spd and NSpd reverted the apparent EPS I deficiencies of the *odc2* and *odc1 odc2* mutants, while the tetraamine Spm only partially complemented the mutants (Fig. S1, available in the online version of this article).

Unlike most *S. meliloti* laboratory strains, strain Rm8530 has a functional *expR* gene that enables it to produce galactoglucan (EPS II), which gives it a very mucoid phenotype on agar plates. By screening for mucoidy on MMS-NH4 plates, we found that the inactivation of *odc2* drastically lowered the amount of ostensible EPS II produced by the mutant (Fig. 1b). A mucoid phenotype was found in the genetically complemented *odc2* mutant *odc2*(pBB5-odc2) (Fig. 1b).

We purified and quantified the total EPS produced by the wild-type and *odc2* strains grown in liquid culture. The masses of total EPS obtained were consistent with the results of the plate assays, and showed that 65% less EPS was produced by the *odc2* mutant (Fig. 1c). EPS production was restored to a wild-type level in *odc2*(pBB-odc2) strain, but not in the

empty vector control strain *odc2*(pBB5). These data indicate that PAs are necessary for normal EPS synthesis in *S. meliloti* Rm8530 (Fig. 1c).

A reduction in intracellular PA content increases H_2O_2 sensitivity

Given the link between bacterial EPS production and oxidative stress resistance [26, 27], we tested to see if PA-deficient *S. meliloti* mutants that produce less EPS are more sensitive to oxidative stress. To do this, we analysed the growth of the *odc1*, *odc2* and *odc1 odc2* mutants in minimal medium containing 0.33 mM H_2O_2. In the H_2O_2-containing medium the *odc1* mutant had a 5% decrease in specific growth rate (μ) and attained the same final OD_{600} as the wild-type. In contrast, the *odc2* and *odc1 odc2* mutants grew with μ values that were 42 and 35 % lower than those of the wild-type, and reached a final OD_{600} that was less than half that of strain Rm8530 (Fig. 2a). Intracellular PAs were present at a normal level in the *odc1* mutant and very low levels in the *odc2* and *odc1 odc2* mutants grown in medium containing H_2O_2 (Fig. 2b, left panel). The specific growth rate of the *odc2* mutant genetically complemented with the *odc2* gene *in trans* was 1.09-fold that of strain Rm8530 (Fig. 2a), and its intracellular PA content was restored to wild-type levels (Fig. 2b, right panel). Introduction of the pBB5 empty vector into the *odc2* mutant resulted in a much lower μ than in the wild-type, an effect that also occurs when the *odc2*(pBB5) strain is grown in minimal medium with or without abiotic stress ([17] and results not shown). This strain had a low level of PAs, similar to the uncomplemented *odc2* mutant (Fig. 2b, right panel).

Decreased motility is correlated with PA deficiency

We found that the swimming motility of the *odc2* mutant was reduced by 40% as compared to the wild-type. A similar motility defect was seen in the empty vector control strain *odc2*(pBB5), while the genetic complementation of the mutant with pBB5-odc2 restored swimming ability to 90% of the wild-type level (Fig. 3a).

Swimming assays were also performed in the presence of exogenous (1 mM) Put, Spd, NSpd and Spm. Put fully restored swimming motility in the *odc2* strain, and increased swimming by the wild-type by 30% with respect to the assay without Put. Surprisingly, the inclusion of 1 mM Spd, NSpd or Spm in the motility assays completely prevented swimming in both strains (Fig. 3b). To determine the concentration dependence of this inhibitory effect, 10- and 100-fold lower concentrations of Spd and NSpd were used in the assays. This showed that the inhibition of swimming ability by Spd and NSpd is concentration-dependent. Exogenous 0.1 and 0.01 mM Spd decreased the motility of Rm8530 by 50 and 30%, respectively, and inhibited the swimming of the *odc2* mutant by 70 and 40%, respectively, compared to the wild-type motility without PAs (Fig. 3c). With NSpd at 0.1 and 0.01 mM swimming by the wild-type strain was inhibited by 80 and 45%, respectively, while the corresponding values for the *odc2* mutant were 95 and 90% versus the wild-type motility without PAs (Fig. 3c).

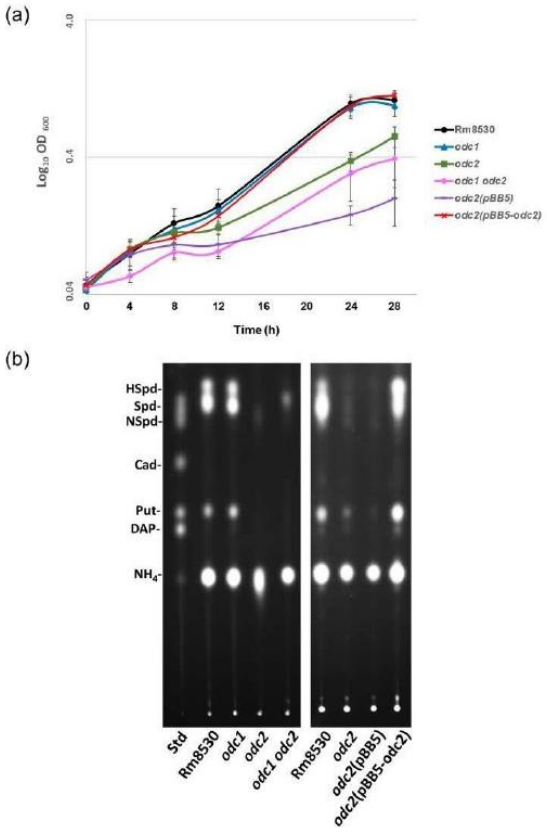

Fig. 2. Growth and polyamine content under oxidative stress conditions (MMS-NH4 with 0.33 mM H_2O_2 added at 0 h). (a) Rm8530 wild-type, black circles; *odc1* mutant, blue triangles; *odc2* mutant, green squares; *odc1 odc2* double mutant, pink diamonds; *odc2* mutant complemented *in trans* with *odc2* gene cloned in pBB5, red Xs; *odc2* mutant containing empty vector pBB5, purple bars. (b) HPTLC detection of dansyl–PAs from 28 h cultures. The lane marked Std contains dansyl–PA standards with their identities shown at the left side of the plate. The *S. meliloti* dansyl–PA samples are identified at the bottom of the figure.

Biofilm formation is increased in the *odc2* mutant

In the microplate assays, the *odc2* mutant formed 25% more biofilm than strain Rm8530 (Fig. 4). To determine how exogenous PAs affect biofilm formation, we performed the assay in the presence of 1 mM Spd, NSpd or Spm. In the wild-type, Spd and NSpd decreased biofilm formation by 20 and 55%, respectively, while in the *odc2* mutant they inhibited biofilm formation by 55 and 70%, respectively, relative to the wild-type without PAs. Spm had the least effect, inhibiting biofilm formation by 30% in the *odc2* strain compared to the wild-type without PAs. Spm did not affect biofilm formation in the wild-type strain (Fig. 4). Biofilm formation in the *odc2* mutant was inhibited to a greater degree than in the wild-type by exogenous Spd and NSpd. Relative to the control culture,

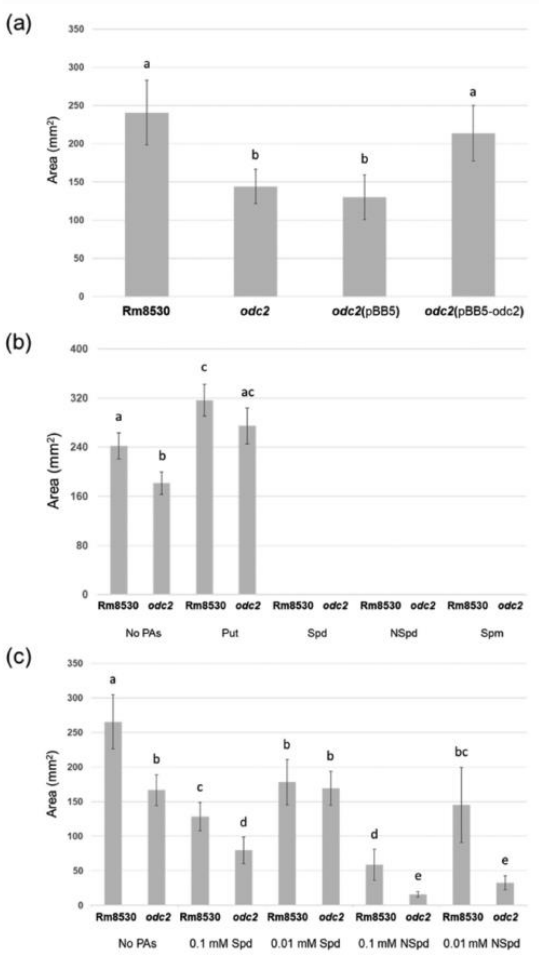

Fig. 3. Swimming motility determined by measuring the diameters of cellular displacement zones in Bromfield medium containing 0.3% agar. (a) Strains are as described in Fig. 1c. The *odc2* mutant and the *odc2* mutant with empty vector pBB5 show less displacement. (b) Complete inhibition of swimming motility in wild-type Rm8530 and the *odc2* mutant by 1 mM exogenous Spd, Nspd and Spm. Note that exogenous Put increases swimming motility versus assays without Put. (c) Effect of exogenous polyamines in micromolar concentrations. Values are the mean±SD for three independent experiments, those with the same letter are not statistically different according to the LSD Fisher test.

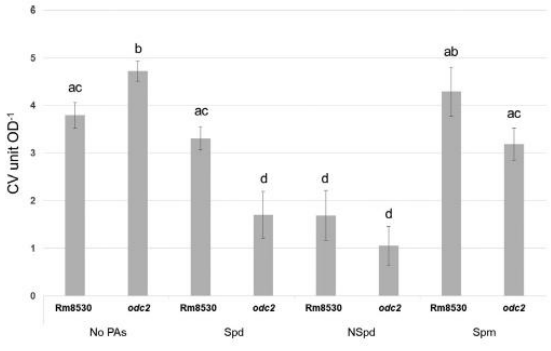

Fig. 4. Biofilm formation by Rm8530 wild-type and the *odc2* mutant as determined by crystal violet staining of cultures grown in 96-well microplates with or without the indicated exogenous polyamine (1 mM). Values are the mean±SD for three independent experiments. The columns marked with the same letter are not statistically different according to the LSD Fisher test.

exogenous Spm reduced biofilm formation by the mutant by about one-third but did not decrease biofilm formation by the wild-type (Fig. 4).

The *odc2* mutant has decreased symbiotic ability

S. meliloti is able to nodulate several species of *Medicago*, *Melilotus* and *Trigonella*, a process that requires motility, biofilm formation, stress resistance and EPS biosynthesis [7]. Because our *in vitro* assays showed that PAs influence physiological processes that are important in symbiosis, we analysed the symbiotic capacity of the Rm8530 *odc2* mutant in combination with *M. sativa*. Forty-five days after the inoculation, the height and dry weight of plants inoculated with the *odc2* strain were diminished by 40%, while the nodule number increased by 50% over the wild-type level (Fig. 5a–c). Most of the nodules formed by *odc2* mutant were white, small and had in total 20% less biomass than those formed on plants inoculated with strain Rm8530 (Fig. 5d). Consistent with these observations, the AR-specific activity of the mutant was 25% lower than that of the wild-type (Fig. 5e). The free PA content in leaves was similar between plants inoculated with the *odc2* and wild-type strains (Fig. 5f, left), while in nodules PA levels were significantly lower in the *odc2* mutant (Fig. 5f, right).

Because the PA decrease in nodules correlates with the reduction in symbiotic efficiency, we evaluated the effect of supplying exogenous Put (1 mM) during symbiosis. In plants inoculated with the *odc2* mutant, exogenous Put restored plant heights to the same level as for wild-type-inoculated plants without exogenous Put (Fig. 5a), while the dry weight was increased by 20% (Fig. 5b). Unexpectedly, exogenous Put increased the number of nodules developed by the mutant by 2.8- and 4.2-fold with respect to the wild-type inoculated plants with and without Put, respectively (Fig. 5c). It should be noted that Put addition also increased the height, dry weight and nodule number in plants inoculated with Rm8530, although it did not increase the nodule biomass or AR specific activity (Fig. 5a–e). Similarly, exogenous Put did not restore AR-specific activity in nodules formed by the *odc2* mutant (Fig. 5e). Although exogenous Put slightly increased the PA content in nodules formed by the *odc2* mutant, their levels remained below those of the wild-type nodules either with and without Put (Fig. 5f, right). This could explain the

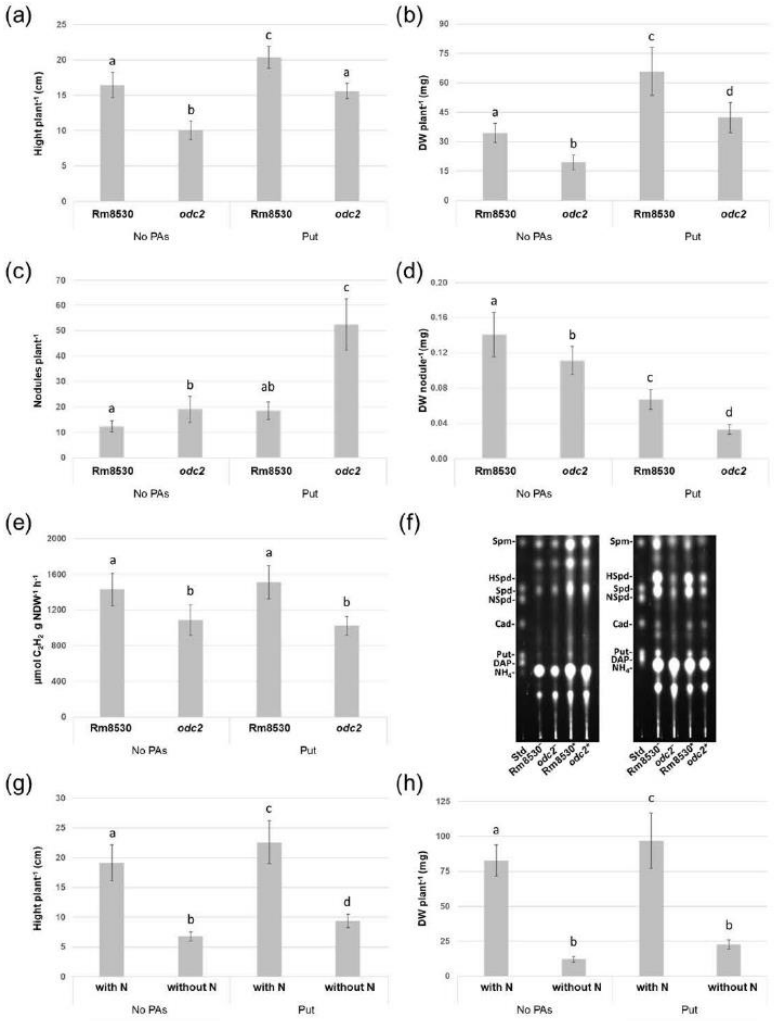

Fig. 5. Symbiotic parameters of the wild-type and *odc2* mutant with alfalfa, with or without (no PAs) supplementation with Put. (a) Plant height. (b) Plant dry weight. (c) Number of nodules per plant. (d) Nodule biomass. (e) Acetylene reduction activity per g dry weight of nodules. (f) PA profiles from leaves (left panel) and nodules (right panel). Lines Rm8530^{-} and *odc2*$^{-}$ represent samples from non-supplemented plants, while lines Rm8530* and *odc2** were treated with Put. (g) and (h) show the effect of supplementation with Put on the height and dry weight, respectively, of uninoculated plants grown with or without nitrogen in the nutrient solution. Values are the mean±SD for three independent experiments. The bars with the same letter are not statistically different according to the LSD Fisher test.

unaltered AR activity of *odc2* and wild-type nodules on Put-treated plants (Fig. 5e). On the other hand, exogenous Put caused a dramatic increase in free PAs in leaves of plants inoculated with the Rm8530 and *odc2* strains (Fig. 5f, left panel), suggesting that Put preferably flows to foliar areas of the plant. The treatment of uninoculated plants with exogenous Put increased both their height (Fig. 5g) and dry weight (Fig. 5h) when watered with either nitrogen-containing or nitrogen-free nutrient solution. The fold-increases in these parameters obtained with Put supplementation of uninoculated plants watered with nitrogen-free nutrient were similar to the increases obtained with plants inoculated with either the wild-type or *odc2* mutant strains (Fig. 5 5a,b), which were also watered with nitrogen-free nutrient.

DISCUSSION

PAs are involved in diverse physiological processes in bacteria, including growth, stress resistance, motility, pathogenesis and biofilm formation. These processes are very interconnected in terms of regulation and physiological consequences. For example, resistance to certain abiotic stresses, pathogenesis and biofilm formation are greatly influenced by EPS production [2, 3, 12].

The ability to produce EPS was greatly reduced in a *S. meliloti odc2* mutant (Fig. 1), which synthesizes significantly less Put, Spd and HSpd than wild-type strain Rm8530 [17]. The results of the calcofluor assay indicate that the mutant produces less EPS I than the wild-type (Fig. 1a). The mutant's decreased ability to produce EPS II was indicated by its less mucoid phenotype (Fig. 1b), while its lower total EPS production was revealed by gravimetric analysis (Fig. 1c).

The ability of Spd, NSpd or Spm to partially or completely restore bright fluorescence (presumably due to EPS I production) to the *odc2* mutant on calcofluor plates indicates a functional redundancy between these PAs. This interpretation is complicated by the fact that the addition of an exogenous PA to *S. meliloti* cultures often affects the intracellular levels of other PAs. In comparison to unsupplemented cultures, cells grown with exogenous Spd have slightly lower levels of Spd, very high levels of Put, a trace of DAP (absent in the control), and no change in HSpd. NSpd-supplemented cultures contain high levels of NSpd (absent in the control), no Spd, traces of Put and HSpd, and a large quantity of DAP [17]. These secondary effects of PA supplementation on PA levels could make an important contribution to their ability to chemically complement the *odc2* mutant.

In *S. meliloti* Rm8530, the ExpR regulator in conjunction with the quorum-sensing system positively regulates genes for EPS I and EPS II production [9, 27]. There are numerous additional positive or negative regulators that modulate the expression of genes involved in the production of either or both EPSs in response to environmental conditions [9]. In addition, the second messenger cyclic dimeric GMP inversely regulates EPS synthesis and motility in *S. meliloti* and many other bacteria [19, 28–31]. This inverse relationship also characterized the *odc2* mutant, which displayed less swimming motility and greater biofilm-forming ability (Figs 3 and 4). Biofilms are formed when one or multiple bacterial species are surrounded by a self-produced polymeric matrix attached to a biotic or inert surface [6, 32]. Biofilm formation involves multiple environmental signals, in addition to structural components such as EPS, lipopolysaccharides and flagella [33, 34]. EPS can comprise 50–90% of the total organic carbon of biofilms [35]. The increased biofilm formation by the *odc2* mutant (Fig. 4) was unexpected, since it produces less total EPS (Fig. 1). Our results are consistent with the mutant being deficient in the production of both EPS I and EPS II, which are important for biofilm formation [29, 36]. The potential production by the mutant of compensatory polysaccharides such as mixed-linkage ß-glucan, which also function in biofilm formation [30, 33], is a question for future research.

Interestingly, Spd, NSpd or Spm supplementation did not restore motility in the *odc2* mutant, but rather had a negative effect on the motility of both this strain and the wild-type (Fig. 3b and c). In *Proteus mirabilis*, Put is able to re-establish the swarming motility of a Put auxotrophic mutant, while Spd, cadaverine (Cad) or agmatine cannot [37].

PAs affect motility and biofilm formation in a variety of bacteria, although the phenomena have not been studied concurrently in the model organisms used [14, 16, 27–41]. We are currently characterizing *S. meliloti* homologues to the *V. cholerae* two-component regulator that modulates cyclic dimeric GMP levels and thus EPS synthesis and biofilm formation in response to endogenous and exogenous PAs [7].

We hypothesize that the greater sensitivity of the *odc2* mutant to oxidative stress is caused by its deficiency in EPS production (Figs 1 and 2). In *Pseudomonas syringae* and *S. meliloti* EPS production is required to contend with H_2O_2 stress [42, 43]. In *S. meliloti*, both EPSs protects against H_2O_2 stress, at least in part by decreasing extracellular H_2O_2 levels [43]. In *Escherichia coli*, PAs also combat oxidative stress by preventing DNA damage and increasing the expression of the genes for catalase (*katG*) and its transcriptional activator *oxyR* [3, 44]. OxyR is well conserved in proteobacteria such as *S. meliloti*, where it regulates the expression of the catalase gene *katA*, the product of which is essential for counteracting oxidative stress [45, 46]. Catalase activity assays of extracts of cells exposed to H_2O_2 shock (2 mM) revealed no differences between the Rm8530 *odc2* mutant and the wild-type (data not shown). This argues that the decreased oxidative stress resistance of the *odc2* mutant comes from it producing less EPS and thus having less ability to lower extracellular H_2O_2 concentrations.

The effective nodulation of legumes by rhizobia requires that they move to the roots, where they initiate processes required for infection, including the synthesis of nodulation factors and EPS, and the formation of biofilms [7, 8, 10]. Because motility, stress resistance, EPS production and biofilm formation are all important in the symbiotic interaction between rhizobia and legumes, it is not surprising that the alteration of these phenotypes in the *odc2* mutant decreased its symbiotic efficiency. Alfalfa plants inoculated with the *odc2* mutant had an increased number of nodules, but lower nodule biomass, AR activity, plant dry weight and plant height (Fig. 5a–e). These parameters, along with the increased number and pale colour of the nodules [47,48], indicate a significantly lowered symbiotic efficiency of the *odc2* mutant.

Exogenous Put restored the height and dry weight of plants inoculated with the *odc2* mutant to those of plants inoculated with the wild-type and grown without added Put. This does not appear to be due to the chemical complementation of the mutant by Put but rather a general effect of Put on plant growth, since comparable increases in growth occurred in uninoculated alfalfa plants grown in nitrogen-free nutrient with added Put (Fig. 5g and h). Alterations in plant hormone levels and/or increased growth caused by exogenous PAs have been reported in alfalfa and other plant species [4, 24, 49–54].

Exogenous Put caused a large increase in free PAs in leaves of plants inoculated with the Rm8530 and *odc2* strains (Fig. 5f, left panel). López-Gómez *et al.* [24] showed that the presence of exogenous Spd and Spm during the *S. meliloti–M. truncatula* interaction led to the accumulation of these PAs in leaves and increased the biomass of root and shoots. In our experiments, Put treatment did not cause accumulation of this PA in leaves but did increase Spd and Spm levels (Fig. 5f, left), indicating that exogenous Put is converted to these PAs by the sequential action of plant Spd and Spm synthases. In nodules formed by the wild-type, Put supplementation did not cause the accumulation in the content of free PAs but rather slightly decreased Spm, HSpd, Spd and Put levels (Fig. 5f, right). This is consistent with the previous finding that HSpd, Cad and Put levels in *M. truncatula* nodules formed by *S. meliloti* decreased in the presence of exogenous Spd and Spm [24].

In summary, we show that alterations in endogenous PA levels affect EPS production, oxidative stress resistance, biofilm formation, motility and symbiotic efficiency in *S. meliloti* Rm8530. Because EPS I and/or EPS II are important or essential components in these processes, we hypothesize that the modulation of EPS production by PAs may be a key determinant of many of the *odc2* mutant's phenotypes. In addition to this, PAs could conceivably affect the expression of specific proteins (principally global regulators) in a manner analogous to what occurs in the *E. coli* polyamine modulon [7].

Funding information
Work was supported by DGAPA-PAPIIT grant IN206317 to M. F. D.

Acknowledgements
V. A. B.-R. is a doctoral student from Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and received fellowship 574042 from CONACyT. A. A is a doctoral student from Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and received fellowship 290118 from CONACyT

Author contributions
V.A. B.-R. formulated the research goals and methodology, performed most of the experiments, analysed data and wrote the draft manuscript. A. A. and A. L. participated in the symbiosis experiments and data analysis. G. H. provided resources and supervised the symbiosis experiments. M. F. D. secured funding, administered and supervised the project, analysed data and wrote the final draft. All authors approved the final draft.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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3. Resultados adicionales

3.1 Crecimiento celular

Con pocas excepciones, las PAs son necesarias para el óptimo crecimiento de diversas bacterias (Michael, 2018). En S. *meliloti* Rm8530, la mutagénesis del gen *smc02983* (mutante *odc2*) reduce notablemente su crecimiento y contenido de HSpd, Spd y Put en medio mínimo (Becerra-Rivera et al., 2018). Sin embargo, aunque en medio rico (Py) sus niveles de Put y HSpd fueron casi abatidos [Fig. 3B], su crecimiento no muestra diferencias con respecto a la cepa wild-type [Fig. 3A]. Probablemente debido a su mediano contenido intracelular de Spd [Fig. 3B], sugiriendo que esta triamina es esencial para el crecimiento normal de S. *meliloti*. No obstante, cabe mencionar que el perfil de PAs en la mutante *odc2* exhibe Spm [Fig. 3B], una tetramina común en eucariontes (Michael, 2016). La cual posiblemente proviene del medio rico (Dunn, 2017) y se desconoce si es capaz de compensar la ausencia de otras PAs.

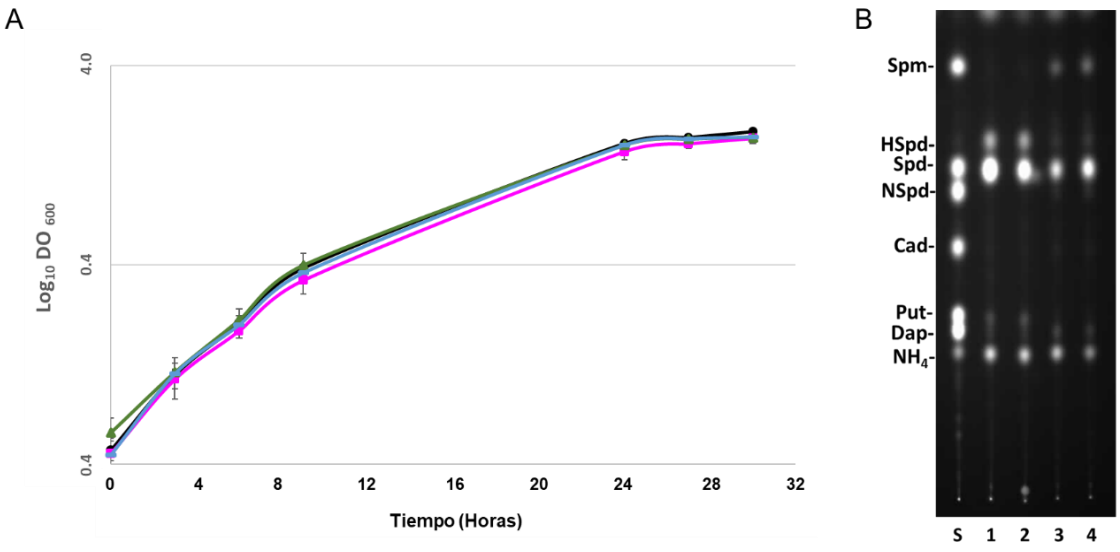

Figura 3. Cinética de crecimiento y contenido de PAs de cultivos de *S. meliloti* Rm8530 en Py. (A) Las cepas wild-type, *odc1*, *odc2* y *odc1 odc2* están representadas por los colores negro, azul, verde y rosa, respectivamente. (B) Detección de PAs dansiladas de cultivos de 24 h por HPTLC. Los carriles en la placa son: S, PAs estándar; 1, Rm8530; 2, *odc1*; 3, *odc2* y 4, *odc1 odc2*.

Consistente con lo observado en medio rico, el defecto en el crecimiento de *odc2* es revertido por la suplementación de Put o Spd (1 mM). Sin embargo, la adición de estos compuestos modificó el contenido de PAs en la cepa wild-type (Becerra-Rivera et al., 2018). En *A. tumefaciens*, una mutante auxotrofa de PAs exhibe una reducción en su crecimiento similar a *odc2*, la cual es restaurada por concentraciones mayores a 1 mM de Put (Kim et al., 2016; Wang et al., 2016).

Para determinar si concentraciones 10 o 100 veces menores a 1 mM son capases de restituir el crecimiento de *odc2* al nivel wild-type, cultivos en medio mínimo fueron tratados con Put exógena al 0.1 y 0.01 mM. Ambas suplementaciones resultaron en un crecimiento especifico normal [Fig. 4A], sugiriendo que transporte de esta diamina en *S. meliloti* es más eficiente que en *A. tumefaciens*. Lo cual podría deberse a la mayor cantidad de trasportadores hipotéticos de Put en *S. meliloti* 1021.

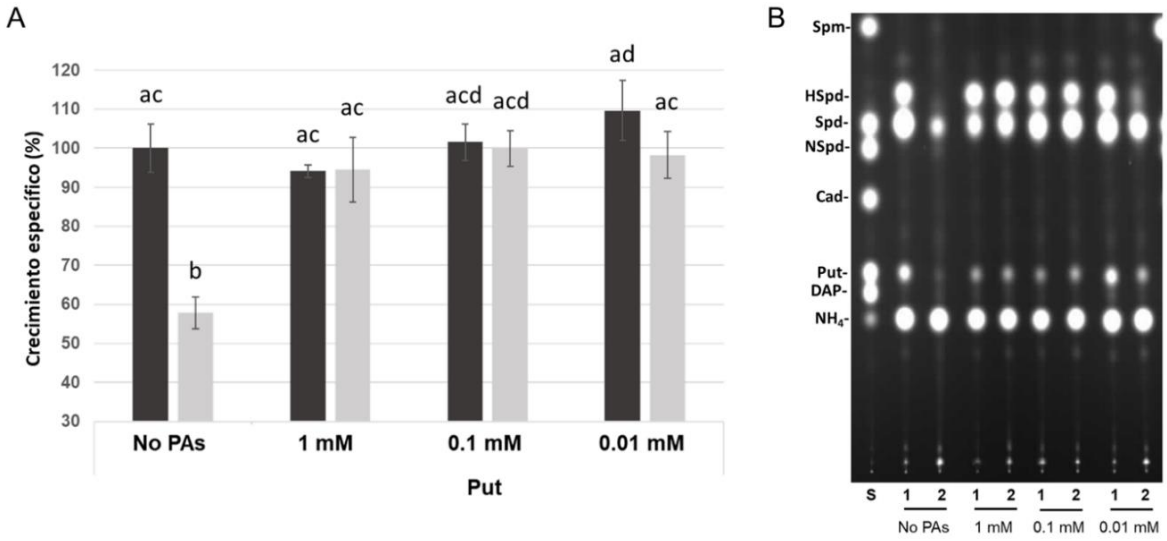

Figura 4. Crecimiento específico (μ, generaciones h^{-1}) y HPTLC de PAs dansiladas de *S. meliloti* con o sin Put exógena. (A) Los valores de μ fueron normalizados al promedio de tres cultivos independientes sin Put (No PAs) de Rm8530, (100% corresponde a 0.169 ± 0.01). Las barras negras y grises indican a las cepas wild-type y *odc2*, respectivamente. (B) Las muestras en la placa fueron purificadas de cultivos de 28 h en medio mínimo con Put exógena y representan a: 1; Rm8530 y 2; *odc2*. S; contiene PAs estándar. Las diferencias significativas fueron determinadas de acuerdo a la prueba de LSD de Fisher.

3.2 Motilidad

Los cambios en los niveles de PAs modifican otros procesos fisiológicos además del crecimiento. Por ejemplo, mientras el μ de Rm8530 es reducido 20% por 1 mM de NSpd exógena, su nado fue completamente abatido. De manera similar, la adición de Spd y Spm (1 mM) inducen el mismo fenotipo en la motilidad (Becerra-Rivera et al., 2018; Becerra-Rivera et al., 2020). Aunque los efectos de concentraciones menores de estas PAs en el crecimiento de *S. meliloti* aun no son analizados. La adición de 0.1 y 0.01 mM de NSpd y Spd revelaron una inhibición dosis dependiente en el nado de las cepas *odc2* y wild-type (Becerra-Rivera et al., 2020).

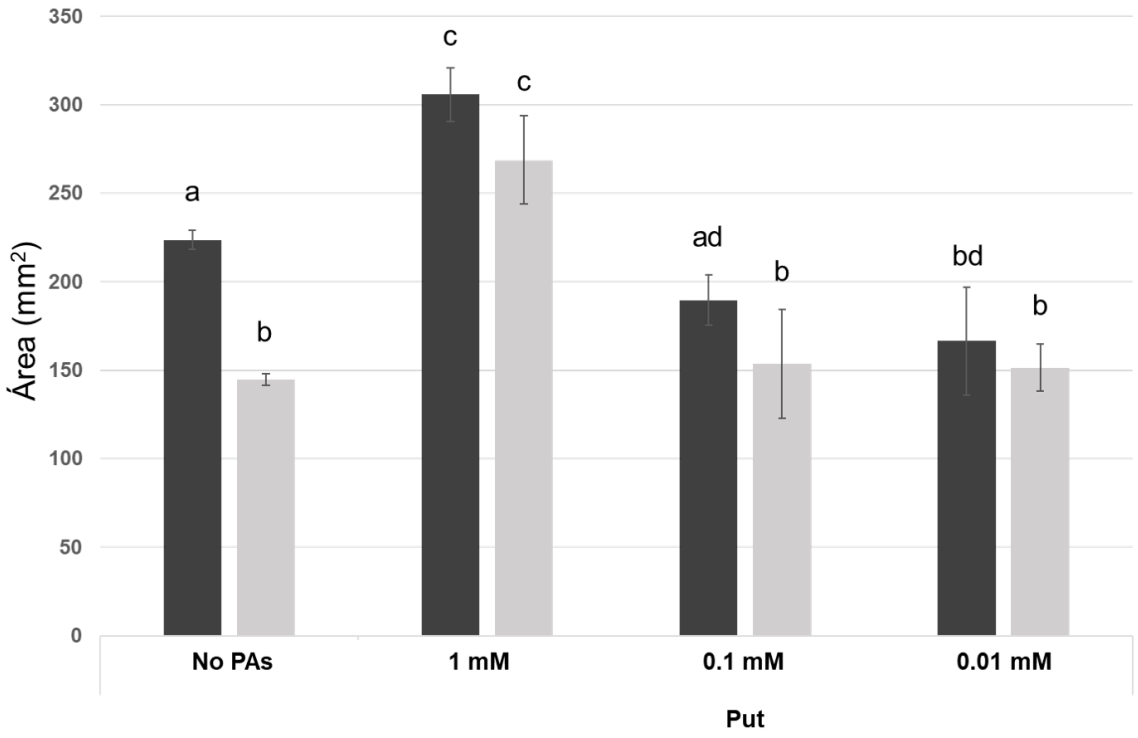

Figura 5. Motilidad de *S. meliloti* Rm8530 en placas de medio semisólido con o sin Put exógena. Las barras negras y grises representan las cepas wild-type y *odc2*, respectivamente. El área promedio fue calculada a partir de tres experimentos independientes. Las concentraciones de Put se indican en figura. De acuerdo con la prueba de LSD de Fisher, las columnas marcadas con la misma letra no muestran diferencias significativas.

Contrario a las triaminas, la suplementación con 1 mM de Put promovió 30% la motilidad de la cepa Rm8530. Mientras en *odc2* además de restaurar su nado, lo incrementa 15% sobre el nivel wild-type (Becerra-Rivera et al., 2020). No obstante, aunque concentraciones menores (0.01 y 0.1 mM) disminuyeron ~20% el nado de Rm8530 [Fig. 5], no mostraron ningún efecto en la cepa mutante. Estos datos demuestran que el papel de la Put en la motilidad depende de su concentración. Un efecto similar fue observado en *D. zeae* donde concentraciones de 0.01 y 0.1 mM de Spd no afectan su nado, mientras 1 mM lo reduce drásticamente (Shi et al., 2019).

Es importante señalar que la adición 0.01 y 0.1 mM de Put exógena resulta en un menor contenido de HSpd en las cepas Rm8530 y *odc2*, en contraste a cultivos wild-type sin PAs. Sin embargo, los niveles de esta diamina incrementan fuertemente en presencia de 1 mM Put [Fig. 4B], dicho cambio podría ser responsable del incremento en su motilidad. Considerando que la HSpd es una de las PAs más comunes en rizobias (Becerra-Rivera & Dunn, 2019), es posible que juegue un papel importante en la fisiología de estas bacterias.

4. Discusión

4.1 Regulación de los niveles de poliaminas en *S. meliloti*

4.1.1 Síntesis de putrescina

En eucariontes, la regulación de los niveles de PAs ha sido bien estudiada en los diferentes niveles, transcripcional, traduccional y postraduccional (Miller-Fleming et al., 2015). No obstante, en bacterias este mecanismo aún continúa describiéndose. En *S. meliloti* Rm8530 la principal ruta de síntesis de Put es mediante la descarboxilación de L-Orn vía ODC2 (Becerra-Rivera et al., 2018), codificada a partir del gen *smc02983*. Este contiene un promotor dependiente de

sigma 70 (Becerra-Rivera & Dunn, 2019) que se expresa fuertemente en medio mínimo y bajo estrés ácido, contario a condiciones de alta salinidad (Domínguez-Ferreras, et al 2006; Becerra-Rivera et al., 2018). Otros factores que regulan negativamente este gen son la Put, Spd y NSpd, las cuales han demostrado inhibir en diferentes niveles la actividad de ODC (Kashiwagi & Igarashi, 1988; Yamamoto et al., 1988).

La ODC2 también es capaz de catalizar la conversión de L-Lys a Cad, pero con menor eficiencia (Becerra-Rivera et al., 2018), similar a las L/ODC de *S. ruminantium* y *V. vulnificus* que exhiben mayor afinidad por L-Orn. Cabe señalar que ambas descarboxilasas son sensibles al inhibidor difluorometilornitina (Takatsuka et al., 2000; Lee et al., 2007). Por el contrario, la adición de este inhibidor no afecta la actividad específica de ODC en células vivas de *S. meliloti*, su contenido de PAs o crecimiento en medio mínimo (Dunn & Becerra-Rivera, datos no publicados), mientras que en *R. leguminosarum* bv. viciae 3841 reduce drásticamente su síntesis de Put y crecimiento celular (Shaw, 2011). Ya que ortólogos de esta proteína están presentes en diferentes rizobias, es probable que sea la enzima clave en la biosisntesis de PAs (Becerra-Rivera & Dunn, 2019).

En *S. ruminantium*, la regulación postraduccional de la L/ODC ha sido descrita detalladamente. La cual requiere la proteína ribosomal L10 que media su degradación a través de una proteasa dependiente de ATP. A pesar de mostrar una secuencia diferente a la antizima eucarionte, L10 promueve la degradación de la ODC de ratón. Tanto la proteína L10 como la antizima eucarionte reconocen el mismo sitio de unión en la L/ODC de *S. ruminantium*, donde los residuos K103, K123 y K126 son esenciales (Yamaguchi et al., 2002; 2006).

En *S. meliloti*, además de encontrar un ortólogo de la proteína L10, también se muestra una similitud en secuencia de aminoácidos de ~40% entre su ODC2 y la L/ODC de *S. ruminantium*, que incluye al residuo K123 mientras K103 y K126 están sustituidos por una L-Ala y L-Arg, respectivamente. Esta última sustitución

es estructuralmente similar a L-Lys y también ocurre en la ODC de ratón (Becerra-Rivera & Dunn, 2019).

Una segunda ODC está presente en *S. meliloti*, la ODC1 es parte de un operón (Schlüter et al., 2013) el cual comprende a los genes *sma0682*, *sma0680*, *sma0678* y *sma0677* (ADC, ODC1, PotE y un transportador hipotético de glutamato/aspartato, respectivamente). Aunque la expresión de estos genes en medio mínimo es alta, la actividad de ODC1 y ADC es significativamente menor en comparación a ODC2 (Arteaga et al., datos no publicados; Becerra-Rivera et al., 2018).

4.1.2 Homoespermidina

Una de las PAs más comunes en rizobias es la HSpd, cuyos niveles responden a diversos factores (Fujihara & Yoneyama, 1993; Fujihara, 2009; López-Gómez et al., 2016b). Por ejemplo, el incremento de la concentración intracelular de Mg^{2+}, que conduce a un mayor contenido de HSpd (Fujihara & Yoneyama, 1994; Fujihara, 2009). En *S. meliloti* Rm8530, los niveles de esta triamina son aumentados casi al doble o reducidos a trazas por Put o NSpd exógenas (1 mM), respectivamente. Bajo condiciones de alta salinidad, el contenido de HSpd es fuertemente reducido en especies de *Sinorhizobium* (Fujihara & Yoneyama, 1993; Becerra-Rivera et al., 2018). Mientras en medio rico, el contenido de esta triamina en *S. meliloti* es menor [Fig. 3B] con respecto a su crecimiento en medio mínimo (Becerra-Rivera et al., 2018).

4.1.3 Biosíntesis de espermidina y norespermidina

Al igual que Spd, la síntesis de NSpd requiere las enzimas CNSpd deshidrogenasa y descarboxilasa, codificadas por los genes *smb21630* y *smb21631*, respectivamente (Dunn, 2017). Sin embargo, esta última triamina también emplea a la DABA aminotransferasa (*rhbA*) y descarboxilasa (*rhbB*) para la biosíntesis de su precursor DAP [Fig. 2]. Transcripcionalmente, *smb21630* es

reprimido por altos niveles de cAMP en *S. meliloti* 2011 (Krol et al., 2016), mientras en condiciones de swarming *rhbA*, *rhbB* y *smb21630* incrementan su expresión (Amaya-Gómez, 2013).

Los niveles de Spd en *S. meliloti* Rm8530 son reducidos ~30% en cultivos suplementados con 1 mM de Put (Becerra-Rivera et al., 2018), mientras en concentraciones 10 y 100 veces menor la síntesis de esta triamina es sumamente activa [Fig. 4B], sugiriendo una inhibición dosis dependiente de la actividad de CNSpd deshidrogenasa y/o descarboxilasa. En *V. alginolyticus*, la actividad de ambas enzimas es reducida ~28% por Spd (Nakao et al., 1991), lo cual correlaciona con la disminución del contenido intracelular de esta y la acumulación de Put durante el crecimiento de *S. meliloti* en presencia de Spd exógena. Contrario a otras PAs, el contenido de Spd se mantiene constante bajo alta salinidad (Becerra-Rivera et al., 2018), a pesar de la sobreexpresión de CNSpd descarboxilasa reportada en la cepa 2011 (Shamseldin et al., 2006).

A diferencia de Spd, la capacidad inhibitoria de NSpd es mayor y reduce la actividad de DABA descarboxilasa, CNSpd deshidrogenasa y descarboxilasa a menos de la mitad (Nakao et al., 1991). Consistente con la fuerte acumulación de DAP y ausencia de Spd en células de *S. meliloti* cultivadas con NSpd exógena (Becerra-Rivera et al., 2018; Becerra-Rivera & Dunn, 2019). Cabe señalar que los niveles de esta triamina en *S. meliloti* están limitados a la fracción ligada a macromoléculas. Sin embargo, en la mutante *odc2* se identificaron trazas de esta poliamina en la fracción libre (Becerra-Rivera et al., 2018).

4.2 Poliaminas en la fisiología de *S. meliloti*

4.2.1 Resistencia a estrés abiótico

Como se mencionó anteriormente, las PAs están asociadas a una amplia gama de procesos fisiológicos en bacterias y otros organismos (Michael, 2016; 2018). En este trabajo se demostró que estas moléculas además de ser

necesarias para el crecimiento de *S. meliloti* Rm8530, son particularmente importantes en la resistencia al estrés abiótico (Becerra-Rivera et al., 2018; Becerra-Rivera et al., 2020).

La resistencia a alta salinidad, acidez y estrés oxidativo es disminuida notablemente en una mutante (*odc2*) con un bajo contenido de PAs libres. La cual reduce ~15% su crecimiento en medio mínimo con 300 mM de NaCl y ~40% a pH 5.5 o con 0.33 mM de H_2O_2 (Becerra-Rivera et al., 2018; Becerra-Rivera et al., 2020). En el primer caso, la menor tolerancia a estrés salino puede ser explicada por la interrupción de un mecanismo homeostático, ligado al nivel de PAs (Fujihara & Yoneyama, 1994; Fujihara, 2009).

En *E. coli* y *S. fredii*, la alta osmolaridad resulta en una disminución de su contenido de Put y HSpd, respectivamente (Munro et al., 1972; Fujihara & Yoneyama, 1993). La cual está asociada a la importación de K^+, exportación de Mg^{2+} y acumulación de L-glutamato. Estos cambios comprenden un mecanismo de equilibrio de cargas que mantiene la electroneutralidad en un medio hipertónico (Fujihara & Yoneyama, 1993; 1994).

Aunque dichos iones no fueron cuantificados en cultivos de *S. meliloti* Rm8530 bajo estrés salino, los niveles de Put y HSpd son fuertemente reducidos (Becerra-Rivera et al., 2018). Sin embargo, en la mutante *odc2* donde la síntesis de PAs es limitada, es probable que este equilibrio de cargas no funcione adecuadamente, ya que la falta de K^+ en un ambiente hipertónico bloquea la exportación de Mg^{2+} y compromete la disminución de HSpd intracelular de *S. fredii* P200 (Fujihara & Yoneyama, 1994). Por lo tanto, es plausible que la ausencia de Put y HSpd en *odc2* [Fig. 3B] repercuta en la importación de K^+ y exportación de Mg^{2+}.

La resistencia a estrés ácido en bacterias es mediada a través de diversos cambios fisiológicos (Kanjee & Houry, 2013). Por ejemplo, la descarboxilación de

L-Lys la cual consume protones citoplasmáticos y se acopla a la importación y excreción de L-Lys y Cad, respectivamente. Permite un ciclo continuo de consumo de H^+, así como la neutralización del medio externo mediante la exportación de Cad (Soksawatmaekhin et al., 2004).

En condiciones ácidas, *S. fredii* P200 incrementa su contenido de HSpd, molécula que ha sido propuesta como un amortiguador que neutraliza los H^+ incorporados a la célula (Fujihara & Yoneyama, 1993), mientras *S. meliloti* Rm8530 mantiene constantes sus niveles de HSpd y Spd (Becerra-Rivera et al., 2018). No obstante, considerando que *S. fredii* no es incapaz de sintetizar Spd (Becerra-Rivera & Dunn, 2019), es probable que esta triamina pueda fungir como un segundo amortiguador en *S. meliloti* durante el estrés ácido, lo cual explicaría porque la mutante *odc2* es más sensible a esta condición (Becerra-Rivera et al., 2018).

Bajo estrés oxidativo, en *E. coli* las PAs promueven la expresión de diferentes genes, incluyendo a los factores transcripcionales OxyR, SoxR y EmrR (Tkachenko & Nesterova, 2003; Sakamoto et al., 2015). El primero está bien conservado en proteobacterias como *S. meliloti*, donde regula la expresión de la catalasa KatA, la cual es esencial para contrarrestar este tipo de estrés (Hérouart et al., 1996; Lehman & Long, 2018). Sin embargo, la actividad de catalasa en extractos de células expuestas a un shock de H_2O_2 no presenta diferencias entre las cepas wild-type y *odc2* (Becerra-Rivera et al., 2020). Por lo tanto, la mayor sensibilidad a H_2O_2 de la mutante *odc2* debe estar asociada a otro mecanismo.

4.2.2 Producción de exopolisacáridos

S. meliloti Rm8530 es capaz de sintetizar el EPS-I o succinoglicano y EPS-II o galactoglucano (Becker et al., 2002), los cuales son importantes para enfrentar diversos tipos de estrés abiótico. Aunque solo el primero ha demostrado tener una importante participación durante la adaptación a pH bajo, además de disminuir los

niveles de H_2O_2 *in vitro*, el EPS-II puede compensar la ausencia del succinoglicano durante el estrés oxidativo (Lehman & Long, 2013, Hawkins et al., 2017).

En la mutante *odc2*, la síntesis de ambos exopolisacáridos es significativamente menor al nivel wild-type. Defecto que es restaurado por la complementación genética, revelando una correlación directa con el contenido de Put, Spd o HSpd y la producción de estos polímeros. Cabe mencionar que el fenotipo de la cepa *odc2* también puede ser revertido por la adición de PAs (Spd y NSpd) propias de *S. meliloti* (Becerra-Rivera et al., 2020).

El EPS-I es un octasacárido constituido por una galactosa y siete residuos de glucosa modificados con grupos succinilo, acetilo y piruvilo, mientras el EPS-II se compone por una galactosa y glucosa. La biosíntesis de ambos exopolisacaridos es un proceso complejo que involucra un extenso grupo de elementos genéticos. Veintidós genes organizados en cinco operones (*wga*, *wgc*, *wggR*, *wgd* y *wge*) y un cluster de 28 genes *exo*/*exs* para el galactoglucano y succinoglicano, respectivamente (Becker et al., 2002; Janczarek, 2015).

En *E. coli*, el modulón de PAs es un conjunto de genes que comprende hasta el momento 20 miembros, cuya traducción es promovida por Put (Igarashi & Kashiwagi, 2015; Sakamoto et al., 2015). Aunque este no ha sido identificado en *S. meliloti*, estas moléculas principalmente forman complejos con el ARN (Igarashi & Kashiwagi, 2015), por lo tanto, es posible que influyan en la síntesis de diversas proteínas.

4.3 Motilidad y formación de biofilm

Una nodulación efectiva requiere que las rizobias se desplacen y colonicen las raíces de leguminosas. Por ende, la motilidad y formación de biofilm son procesos de suma importancia (Poole et al., 2018). En la mutante *odc2*, el nado es fuertemente reducido, mientras en la cepa Rm8530 la adición de Spd y NSpd

exógenas lo inhiben de manera dosis dependiente (Becerra-Rivera et al., 2020). Aunque la diamina Put también es capaz de inhibir la motilidad, su efecto es considerablemente menor y está limitado a concentraciones micromolares, ya que en dosis milimolar restablece y promueve el nado de las cepas *odc2* y wild-type, respectivamente [Fig. 5].

Contrario a los patógenos *P. mirabilis* y *D. zeae* cuya motilidad depende de Put (Sturgill & Rather, 2004; Shi et al., 2019), el nivel de PAs en la mutante *odc2* reveló que concentraciones bajas de esta diamina son dirigidas a la síntesis de Spd, mientras concentraciones mayores parecen inhibir esta vía y redirigirla a la biosíntesis de HSpd [Fig. 4B]. Sugiriendo que esta triamina es clave para el nado en *S. meliloti*, similar a lo reportado en *R. etli* CNPAF512 donde la HSpd es necesaria para su motilidad (Braeken et al., 2008).

En *S. meliloti*, la motilidad y formación de biofilm son mecanismos opuestos (Amaya-Gómez et al., 2015), lo cual es consistente con el incremento en la formación de biofilm en la mutante *odc2* (Becerra-Rivera et al., 2020). En diversas bacterias, las PAs son capaces de modificar este proceso. Por ejemplo, en *A. tumefaciens* C58 la formación de biofilm es aumentada cuatro veces en una mutante autótrofa de PAs (*Δodc*) (Wang et al., 2016), mientras en *V. cholerae* su producción es regulada por NSpd, Spm o Spd (Cockerell et al., 2014).

La adición de Spd y NSpd exógenas reduce significativamente el biofilm sintetizado por *odc2* (Becerra-Rivera et al., 2020). De manera similar, la Spd previene la formación de biofilm y evita su sobreproducción en *V. cholerae* y *Δodc*, respectivamente (Cockerell et al., 2014; Wang et al., 2016). En ambos organismos, la formación de biofilm es directamente proporcional a la síntesis de polisacáridos, mientras en la mutante *odc2* de *S. meliloti* Rm8530 la producción de EPS-I y II es menor (Cockerell et al., 2014; Wang et al., 2016; Becerra-Rivera et al., 2020).

4.4 Simbiosis

La nodulación es un proceso complejo que requiere un constante dialogo molecular entre planta y bacterias (Poole et al., 2018). *S. meliloti* es un microendosimbionte de distintas especies de leguminosas, incluyendo *M. sativa* (alfalfa). En la cual, a diferencia de otras plantas los defectos simbióticos causados por la ausencia del EPS-I son revertidos por el EPS-II (Leigh et al., 1985; Glazebrook & Walker, 1989). Consistente con esto, plantas infectadas con la cepa *odc2* reducen su actividad de nitrogenasa, peso seco y altura (Becerra-Rivera et al., 2020).

Por el contrario, el número de nódulos formados por la mutante incrementó drásticamente, aunque su tamaño y biomasa fue menor, incluso exhibieron una coloración blanquecina (Becerra-Rivera et al., 2020), características típicas en nódulos ineficaces (Hardarson et al., 1981; Singleton & Stockinger, 1983; Regensburger et al., 1986; Glazebrook & Walker, 1989). El contenido de PAs en estas estructuras, fue fuertemente reducido en contraste a nódulos formados por la cepa Rm8530, mientras que en hojas no se observaron diferencias (Becerra-Rivera et al., 2020). Una mutante en HSS de *R. tropici* CIAT899, produce nódulos con biomasa disminuida, sugiriendo un defecto en la organogénesis asociado a la ausencia de HSpd (López-Gómez et al., 2016b).

La suplementación con Put, restauró la altura y peso en plantas infectadas con *odc2*. Sin embargo, también promovió la altura y biomasa en plantas inoculadas con la cepa wild-type (Becerra-Rivera et al., 2020), indicando que las PAs en alfalfa pueden estar involucradas en su crecimiento. Diversos estudios han demostrado que las PAs provocan alteraciones en los niveles hormonales de las plantas (Palma et al., 2013; 2014; Radhakrishnan & Lee, 2013; López-Gómez et al., 2016a). Por ejemplo, la Spd producida por *B. subtilis* OKB105 disminuye el nivel de etileno e induce la expresión de genes de expansina en tabaco. Mientras la primera inhibe el alargamiento de la raíz en numerosas plantas, las proteínas de

expansina promueven la división y extensión celular, estimulando el crecimiento vegetal (Choi et al., 2003; Xie et al., 2014).

Sorprendentemente, aunque la Put exógena aumento el número de nódulos y redujo su biomasa, no alteró la actividad de nitrogenasa en plantas infectadas con la cepa *odc2* o con Rm8530. El análisis del contenido de PAs en nódulos formados por la mutante, reveló un ligero incremento. No obstante, este continuó por debajo del nivel wild-type (Becerra-Rivera et al., 2020). Lo cual es consistente con López-Gómez et al. (2016b), quienes reportaron una directa correlación entre mayor fijación de nitrógeno y altos contenidos de Put, Spd y Spm en bacteroides de *R. tropici* en frijol.

En nódulos wild-type, la complementación química provocó una sutil reducción en los niveles de Spm, HSpd, Spd y Put (Becerra-Rivera et al., 2020). Similar a lo observado en nódulos formados por *S. meliloti* en *M. sativa*, donde su contenido de HSpd, Cad y Put es disminuido en presencia de Spd y Spm exógenas. Por el contrario, este tratamiento demostró acumulación de ambas traminas en hojas, así como mayor biomasa en raíz y brotes (López-Gómez., 2017). En plantas inoculadas con la mutante *odc2* y la cepa Rm8530, el tratamiento con Put no mostró acumulación de esta diamina en hojas, aunque aumentó los niveles de Spd y Spm (Becerra-Rivera et al., 2020), sugiriendo que la Put exógena es transformada a estas triaminas mediante las sintasas de Spd y Spm de la planta, además de un flujo preferencial hacia el área foliar.

5. Conclusión

La biosíntesis de PAs en *S. meliloti* Rm8530 es mediada principalmente por una L/ODC (ODC2). Al igual que en muchos procariontes, cambios en los niveles de estas moléculas afectan su crecimiento, resistencia a estrés abiótico, producción de exopolisacáridos, motilidad y el desarrollo de una simbiosis efectiva

en alfalfa. Por el contrario, un bajo contenido de PAs promueve la formación de biofilm [Fig. 6].

Aunque algunos de los fenotipos observados (tolerancia a estrés, formación de biofilm o simbiosis) pueden atribuirse a la alteración en la síntesis de exopolisacáridos, las PAs han demostrado ser capases de modificar la expresión de numerosos reguladores transcripcionales en *E. coli* (Igarashi & Kashiwagi, 2015; Sakamoto et al., 2015). Por lo cual, investigar si mecanismos similares ocurren en *S. meliloti* es esencial.

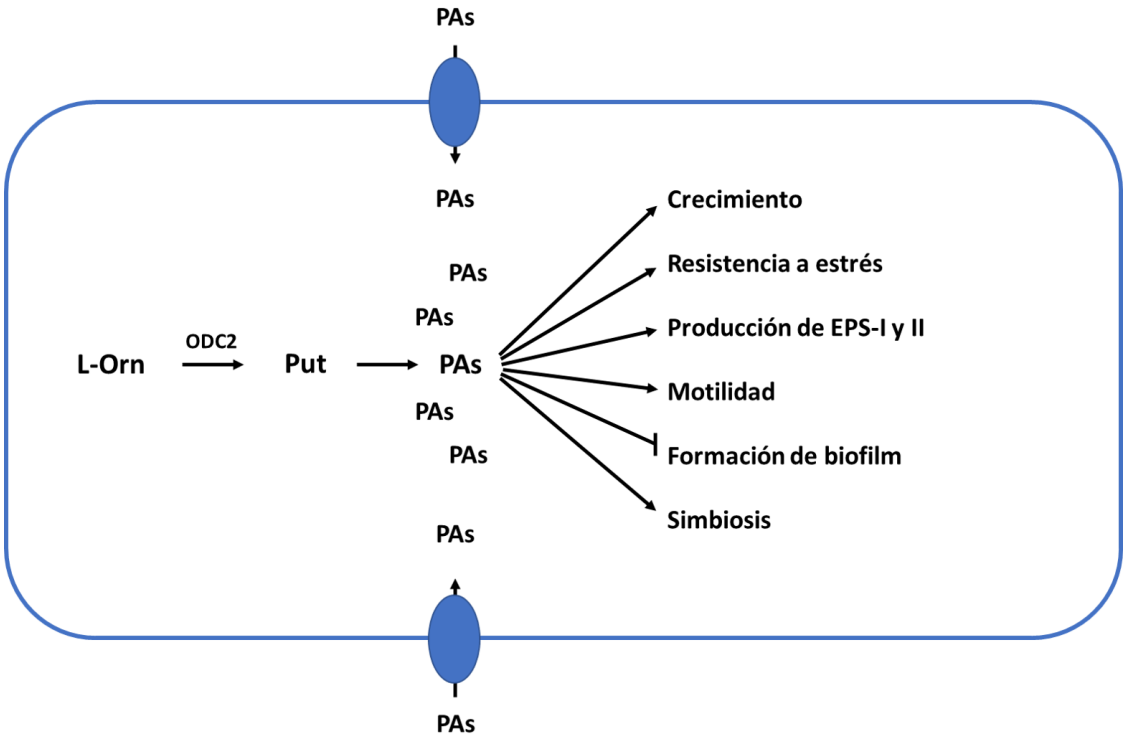

Figura 6. Biosíntesis y roles fisiológicos de las PAs en *S. meliloti* Rm8530. El esquema muestra la enzima clave (ODC2) en la síntesis de PAs. Diversos procesos requieren niveles intracelulares óptimos de estas moléculas (→), contrario a la formación de biofilm (⊣).

6. Perspectivas

Identificar cambios potenciales en la trascripción y/o traducción entre las cepas wild-type y *odc2*.

Caracterizar el hipotético sistema sensor de PAs, NspS/MbaA.

Asignar PAs especificas a procesos particulares.

7. Anexo

FEMS Microbiology Letters, 366, 2019, fnz084

doi: 10.1093/femsle/fnz084
Advance Access Publication Date: 23 April 2019
Minireviews

MINIREVIEWS – Physiology & Biochemistry

Polyamine biosynthesis and biological roles in rhizobia

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One sentence summary: Synthesis pathways and physiological functions of polyamines in free-living and symbiotically associated rhizobia are reviewed.

Editor: Hermann Bothe

ABSTRACT

Polyamines are ubiquitous molecules containing two or more amino groups that fulfill varied and often essential physiological and regulatory roles in all organisms. In the symbiotic nitrogen-fixing bacteria known as rhizobia, putrescine and homospermidine are invariably produced while spermidine and norspermidine synthesis appears to be restricted to the alfalfa microsymbiont *Sinorhizobium meliloti*. Studies with rhizobial mutants deficient in the synthesis of one or more polyamines have shown that these compounds are important for growth, stress resistance, motility, exopolysaccharide production and biofilm formation. In this review, we describe these studies and examine how polyamines are synthesized and regulated in rhizobia.

Keywords: polyamines; rhizobia-legume symbiosis; nitrogen fixation; basic amino acid decarboxylases; *Sinorhizobium meliloti*

INTRODUCTION

Polyamines are aliphatic hydrocarbons containing two or more amino groups, which are positively charged at physiological pH. They are ubiquitous in the three domains of life and have important or essential roles in growth, cell viability, transcription, translation, pathogenesis, biofilm formation, stress resistance and motility. Polyamines participate in these processes either in free form or by interacting with and modifying the functional properties of macromolecules like nucleic acids, proteins and phospholipids. For example, spermidine (Spd) is essential in eukaryotes and archaea because its aminobutyl moiety is used to chemically modify translation factor eIF5A, which lets the ribosome translate polyproline tracts encoded in mRNA. Other functions of polyamines in these domains include the essential chemical modification of tRNA(Ile) in archaea, embryonic development in mammals, stress resistance and embryo development in *Arabidopsis thaliana*, and full mental development in humans (Childs, Mehta and Gerner 2003; Bachrach 2005; Rhee, Kim and Lee 2007; Shah and Swiatlo 2008; Michael, 2015, 2016; Miller-Fleming *et al.* 2015; Pegg 2016; Lenis *et al.* 2017; Handa, Fatima and Matoo 2018).

Unlike their roles in archaea and eukaryotes, polyamines in bacteria have no universal essential role but have important functions that are much more diverse and often species specific (Michael, 2016, 2018). This is illustrated by the widely varying requirement for polyamines for the growth of different species. The pathogens *Enterococcus faecalis* and *Staphylococcus aureus* neither produce nor require polyamines for growth (Li *et al.* 2019). In contrast, the great majority of bacteria produce one or more different polyamines and studies with polyamine biosynthesis mutants indicate their relative importance for growth. *Pseudomonas aeruginosa*, *Agrobacterium tumefaciens* and *Campylobacter jejuni* absolutely require polyamines for growth (Michael 2018). Mutants of *Escherichia coli* unable to make polyamines grow at about half the wild type rate under aerobic conditions, but do

Received: 27 February 2019; **Accepted:** 22 April 2019

not grow under anaerobic conditions (Chattopadhyay, Tabor and Tabor 2009). In *E. coli*, polyamines affect growth by facilitating ribosome assembly and increasing the efficiency of protein synthesis. They also increase the translation of several global transcriptional regulators (the polyamine modulon) that have profound effects on gene expression and metabolism (Igarashi and Kashiwagi, 2006, 2018).

Rhizobia are a diverse group of α- and β-proteobacteria that reduce nitrogen to ammonia in symbiosis with specific leguminous host plants (Poole, Ramachandran and Terpolilli 2018). The group includes nitrogen-fixing genera such as *Rhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Bradyrhizobium*, and non-nitrogen fixing plant pathogens in the genus *Agrobacterium* (Dunn 2015).

To establish the symbiosis, rhizobia move by chemotaxis to host roots, respond to plant-exuded flavonoids by synthesizing lipochitooligosaccharide nodulation (Nod) factors and exopolysaccharides (EPS), and form biofilms on the host roots. Rhizobia infect the host through root hairs while the root cortex differentiates into the nodule structure in response to the Nod factors. The rhizobia invade plant cells in the nodule and metabolically differentiate into their microaerobic, nitrogen-fixing form, called bacteroids. The bacteroid nitrogenase complex reduces nitrogen to ammonia, which is exported to the plant in exchange for plant-derived carbon sources (Haag *et al.* 2013; Poole, Ramachandran and Terpolilli 2018).

Both rhizobia and pathogens share traits that are important for their interaction with hosts, including growth capacity, abiotic stress resistance, motility, EPS production and biofilm formation. We describe here how some of these qualities are influenced by polyamines and some possible mechanisms for these effects. We also describe how polyamine synthesis occurs and is regulated in rhizobia.

POLYAMINE BIOSYNTHESIS AND PHYSIOLOGICAL FUNCTIONS

Here, we first consider how polyamines are synthesized by different rhizobial species. Intracellular polyamines exist free in the cytoplasm or ligated to other molecules. The free polyamines are soluble in the weak acid solutions used to extract them from cells in the laboratory. In contrast, bound polyamines linked to nucleic acids, proteins, or cell wall constituents precipitate along with these components in the acidic extract. The bound polyamines can be obtained in free form and then identified following strong acid hydrolysis of the precipitate from the acidic extract (Dunn 2017).

The polyamine biosynthesis pathway in *E. coli* (Fig. 1) allows the production of three different polyamines (Chattopadhyay, Tabor and Tabor 2009), while that of *Vibrio cholerae* results in five different polyamines. The *Sinorhizobium meliloti* pathway (Fig. 2) is identical to that of *V. cholerae* except that the latter organism does not synthesize homospermidine (HSpd) (Tower 1987; Lee *et al.* 2009; Becerra-Rivera *et al.* 2018).

The principal free polyamines found in rhizobia are the diamine putrescine (Put) and the triamine HSpd. In addition, cadaverine (Cad) and Spd occur in some strains of *S. meliloti* and *Bradyrhizobium japonicum* (Hamana, Minamisawa and Matsuzaki 1990; Auling *et al.* 1991; Dunn 2017; Fig. 1). Aminobutylhomospermidine (AbuHSpd; $H_2N(CH_2)_4NH(CH_2)_4NH(CH_2)_4NH_2$) is found in many fast-growing rhizobia but is absent in slow growing species like *B. japonicum* (Fujihara and Harada 1989).

The free polyamines identified in representative rhizobia (Fig. 3) are consistent with the predicted complement of polyamine biosynthesis genes in these species (Table 1). All six species produce Put and HSpd, while *S. meliloti* additionally produces Spd. Norspermidine (NSpd; Fig. 2) is also unique to *S. meliloti* and is present only in the bound polyamine fraction, which also appears to contain Cad. 1,3-diaminopropane (DAP) is detectable in the *S. meliloti* free polyamine fraction when the cells are grown in the presence of exogenous NSpd (Becerra-Rivera *et al.* 2018). In *Vibrio alginolyticus*, NSpd inhibits the activities of carboxynorspermidine dehydrogenase (CANSDH, EC 1.5.1.43) and carboxynorspermidine decarboxylase (CANSDC (NspC), EC 4.1.1.96), two enzymes of the NSpd synthesis pathway that occur after DAP formation (Nakao, Shinoda and Yamamoto 1991). The inhibition of these enzymes (Fig. 2) in NSpd-supplemented *S. meliloti* cultures is probably the cause of DAP accumulation (Becerra-Rivera *et al.* 2018).

HSpd is often the most abundant polyamine in rhizobial bacteroids or free-living cells. The tetraamine spermine (Spm) is rare in bacteria and is not present in nitrogen-fixing rhizobia unless they are grown in rich medium, which contains Spm that is taken up by the cells (Busse and Auling 1988; Hamana, Minamisawa and Matsuzaki 1990; Auling *et al.* 1991; Fujihara *et al.* 1994; Hosoya *et al.* 2004; López-Gómez *et al.* 2016). Spm is, however, made by some *Agrobacterium tumefaciens* strains grown in minimal medium, along with Put, Spd and HSpd (Hamana, Minamisawa and Matsuzaki 1990; Kim *et al.* 2016).

Polyamine biosynthesis begins with reactions catalyzed by monofunctional ornithine (Orn), arginine (Arg) or lysine (Lys) decarboxylases (ODC, ADC and LDC, respectively) or bifunctional enzymes that decarboxylate both Orn and Lys (L/ODC). The distribution, biochemistry and regulation of these pyridoxal 5'-phosphate-dependent enzymes has been reviewed (Kidron *et al.* 2006; Michael 2015).

Among rhizobia, the biosynthesis of the polyamine precursors Arg, Orn and Lys has been studied in *S. meliloti*, which appears to have a particularly high metabolic capacity for Orn synthesis (Dunn 2015; Hernández *et al.* 2015). Put is produced directly from Orn by ODC (EC 4.1.1.17), which is present in many bacteria including *E. coli* (Fig. 1). In rhizobia, ODCs occur only in *S. meliloti*, *Rhizobium tropici* and *B. japonicum*, while putative L/ODCs (EC 4.1.1.18/EC 4.1.1.17) are present in all of the species considered here (Table 1). Growing *R. leguminosarum* bv. viciae 3841 in the presence of the ODC inhibitor difluormethylornithine (DFMO) reduced its levels of Put and HSpd by 95% and significantly decreased its growth. Exogenous Put added to the DFMO-treated cultures restored normal levels of polyamines and growth (Shaw *et al.* 2010). An *A. tumefaciens* C58 ODC deletion mutant synthesized only trace levels of Put and Spd, had a slower than wild type growth rate and yield, and formed more biofilm. Normal growth and biofilm phenotypes were restored in cultures containing exogenous Put or Spd (Kim *et al.* 2016; Wang *et al.* 2016).

The *S. meliloti* Rm8530 L/ODC is designated ODC2 (Table 1) and has 10-fold higher activity with Orn as substrate (forming Put) as compared to Lys (forming Cad) (Becerra-Rivera *et al.* 2018). It belongs to the same ODC subfamily as eukaryotic ODCs (EC 4.1.1.17) and bacterial L/ODCs like that from *Selenomonas ruminantium* (Takatsuka *et al.* 2000). An *odc2* null mutant makes only trace levels of Put, Spd and HSpd and grows significantly slower than wild type in succinate-ammonium minimal medium, especially under salt or acidic stress. The mutant grows like wild type in cultures containing exogenous Put or Spd (Becerra-Rivera *et al.*

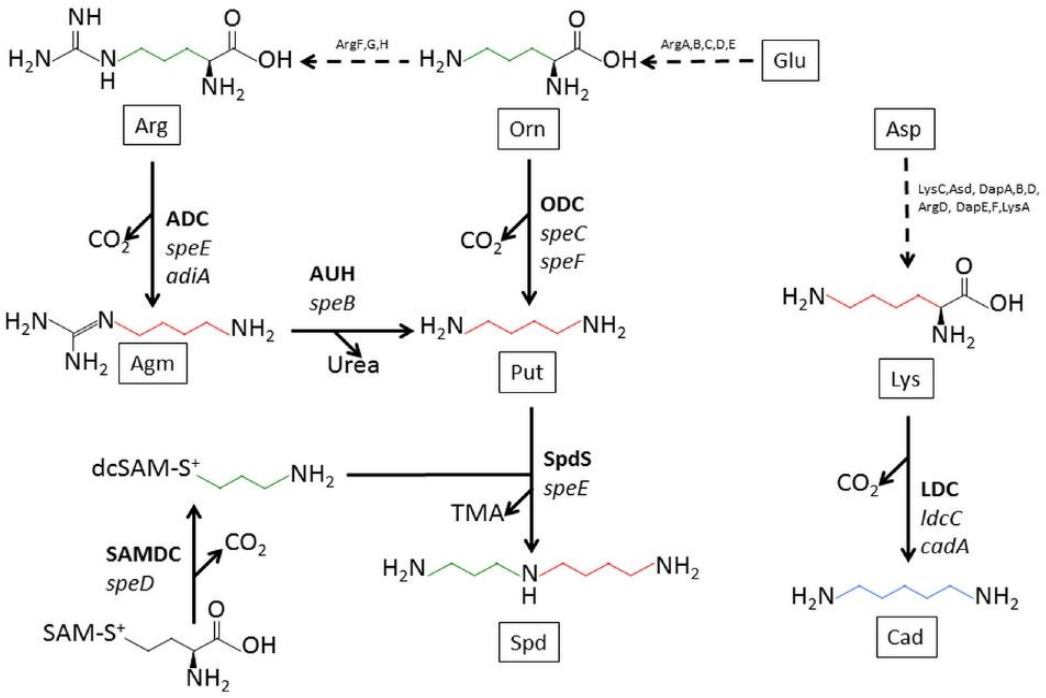

Figure 1. Polyamine biosynthesis pathways in *E. coli*. For the basic amino acid decarboxylases, ODC, LDC and ADC, the constitutive and inducible forms are respectively encoded by: ODC, *speC* and *speF*; LDC, *ldcC* and *cadA*; ADC, *speA* and *adiA*. Carbon chains are color coded to highlight the number of carbons between amino groups, as follows: green, 3 carbons; red, 4 carbons; blue, 5 carbons. Abbreviations not defined in the text: Asp, L-aspartate; dcSAM, decarboxylated S-adenosylmethionine; Glu, L-glutamate; SAM, S-adenosylmethionine; SAMDC, S-adenosylmethionine decarboxylase; TMA, thiomethyladenosine; SpdS, spermidine synthase.

Table 1. Polyamine biosynthesis genes in Rhizobia. These results are based on both bioinformatic analysis of rhizobial genome sequences and functional analysis of some of the gene products (see the main text) .

	Organism[a]						
Gene[b]	*S. meliloti*	*S. fredii*	*R. etli*	*R. leguminosarum bv. viciae*	*R. tropici*	*M. loti*	*B. japonicum*
baadc	SMa0682				CH15460	Mll1584	
odc	SMa0680 (ODC1)				PC09200		Bll3177
l/odc	SMc02983 (ODC2)	NGR_c29260	RHE_CH03629	RL4156	PC01140	Mll2974	Blr7759
auh	SMc01967	NGR_c25370	RHE_CH02143	pRL100366 RL1669	CH06650 (SpeB)	Mll4895	
	SMc01802	NGR_c10200	RHE_CH01568		PC06540 (SpeB) PC07825 (SpeB)	Mll8453	
hss	SMc04016	NGR_c2847	CH03553	RL4156	CH15075	Mlr3014	Blr7762
cansdh	SMb21630						
cansdc	SMb21631						
dabaat	SMa2400 (RhbA)						
dabadc	SMa2402 (RhbB)						

[a]The strains are *S. meliloti* 1021, *S. fredii* NGR234, *R. etli* CFN42, *R. leguminosarum* bv. viciae 3841, *R. tropici* CIAT899, *M. loti* MAFF303099; *B. japonicum* USDA110.
[b]Abbreviations: *baadc*; basic amino acid decarboxylase (these are often annotated as Orn/Lys/Arg, Arg/Lys/Orn or Orn/diaminopimelate/Arg decarboxylases); *odc*, Orn decarboxylase; *l/odc*, bifunctional Lys/Orn decarboxylase; *auh* and SpeB, agmatine ureohydrolase (agmatinase); *hss*, HSpd synthase; *cansdh*, carboxynorspermidine dehydrogenase; *cansdc*, carboxynorspermidine decarboxylase; *dabaat*, DABA aminotransferase; *dabadc*, DABA decarboxylase.

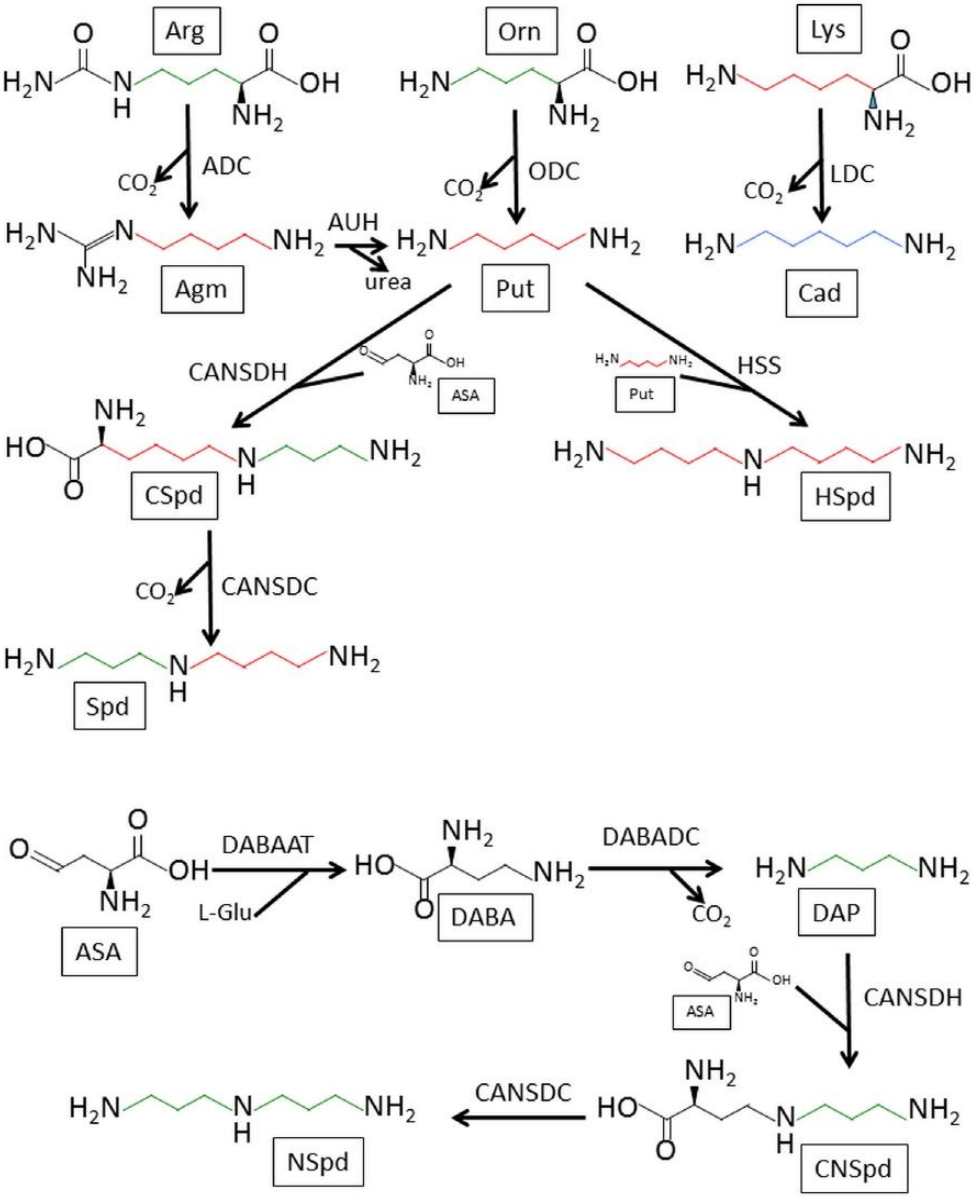

Figure 2. Polyamine biosynthesis pathways in *S. meliloti* 1021. The top of the figure shows the polyamines or intermediates produced by the decarboxylation of basic amino acids and the production of HSpd and Spd from Put. Note that experimental evidence for ADC activity is lacking. The lower part of the figure shows the NSpd biosynthesis pathway. The color coding of carbon chains is as for Fig. 1.

2018). A transposon mutagenesis followed by sequencing (Tn-Seq) study in *S. meliloti* 1021 showed that an *odc2* mutant had deficient growth on many of the carbon and nitrogen sources tested (Price *et al.* 2018). A second Tn-seq study showed that *odc2* was important for colonizing the roots of both a non-host (rice) and a host (*Medicago truncatula*) plant (VanYperen 2015). *S. meliloti* has a monofunctional ODC designated ODC1 (SMa0680) that decarboxylates Orn with a specific activity about 10% that of ODC2. An *odc1* null mutant is indistinguishable from the wild type in terms of polyamine production and growth (Becerra-Rivera *et al.* 2018).

The decarboxylation of Arg by ADC (EC 4.1.1.19) produces Agm, which is hydrolyzed to Put by agmatinase (agmatine ureohydrolase, AUH or SpeB, EC 3.5.3.11) (Fig. 2). ADC occurs in *E. coli* (Fig. 1), *B. subtilis* and *V. cholerae* (Lee *et al.* 2009; Burrell et

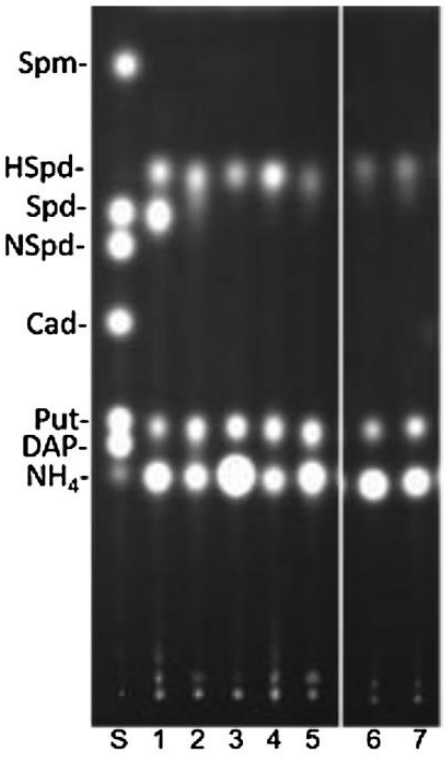

Figure 3. Soluble intracellular polyamines present in rhizobia grown in minimal medium. Polyamines were extracted, derivatized with dansyl chloride, resolved by thin layer chromatography and photographed under UV light. Lane S, dansyl-polyamine standards, which are identified to the left of the lane. HSpd is not commercially available but the identity of dansyl-HSpd has been unambiguously established (Becerra-Rivera *et al.* 2018). Lane 1, *S. meliloti* 1021; lane 2, *S. fredii* NGR234; lane 3, *B. japonicum* USDA110; lane 4, *M. loti* MAFF303099; lane 5, *R. tropici* CIAT899; lane 6, *R. leguminosarum* bv. viciae 3841; lane 7, *R. etli* CFN42.

al 2010; Hobley *et al.* 2017). While there is no experimental evidence for ADCs in rhizobia, *S. meliloti*, *R. tropici* and *Mesorhizobium loti* have genes designated as basic amino acid decarboxylases (*baadc*) that could potentially encode ADCs (Table 1). Consistent with this, *R. tropici* was reported to have low ADC activity in addition to high ODC activity (López-Gómez *et al.* 2016), the former possibly arising from the product of its *baadc* (Table 1). Agm, the product of ADC activity, has not been detected in rhizobia (Fujihara 2009) but AUH, which uses Agm as substrate, was detected in *S. meliloti* 1021 (Díaz *et al.* 2011). However, the possible presence of AUH in other rhizobia must be inferred from gene annotations or sequence comparisons, and it is often difficult to distinguish AUH from other members of the ureohydrolase superfamily, such as arginase. With this caution in mind, we observe that most rhizobia appear to encode two or more AUHs (SpeBs) despite their apparrent lack of ADC (Table 1). One of the two putative *speB* homologs invariably neighbors the gene encoding *N*-acetylglutamyl phosphate reductase (ArgC; EC 1.2.1.38), part of the Arg synthesis pathway. The *S. meliloti speB* and *argC* genes are transcribed from separate promoters and so could be independently regulated (Díaz *et al.* 2011; Dunn 2017). The genes for the second possible AUH (*speB2*) in *S. meliloti* and *S. fredii* lie next to those encoding a putative PotABCD transporter for Spd and Put.

In eukaryotes, Spd is formed by transferring the aminopropyl group from decarboxylated *S*-adenosylmethionine (dcSAM) to Put in a reaction catalyzed by Spd synthase (EC 2.5.1.16) (Fig. 1). Spd can be aminopropylated by Spm synthase (EC 2.5.1.22) using dcSAM to produce Spm (Pegg 1986; Shah and Swiatlo 2008; Michael 2015). The Spd synthase pathway is common in bacteria while the existence of Spm synthase remains to be confirmed (Michael 2015).

Some bacteria have an 'alternative' Spd synthesis pathway that does not involve Spd synthase (Lee *et al.* 2009). This pathway (Fig. 2) catalyzes the reductive condensation of Put and L-aspartate ß-semialdehyde (ASA) to form carboxyspermidine (CSpd) in a NADPH-dependent reaction catalyzed by CANSDH. CSpd is then decarboxylated by CANSDC to form Spd. In *S. meliloti cansdh* and *cansdc* form a 2-gene operon on plasmid pSymB.

In *S. meliloti*, NSpd synthesis starts with the conversion of L-aspartate ß-semialdehyde and L-glutamate to 1,4-diaminobutyric acid (DABA) in a reaction catalyzed by DABA aminotransferase (DABAAT (RhbA), EC 2.6.1.76). DABA is decarboxylated to DAP by DABA decarboxylase (DABADC (RhbB), EC 4.1.1.86) (Fig. 2). The DAP produced by these enzymes is also used for the synthesis of the siderophore rhizobactin 1021. The *rhbA* gene is first in a 6 gene *rhb* operon that contains other rhizobactin 1021 biosynthesis, transport and regulatory genes. The insertion of Tn*5lac* into the *S. meliloti* 2011 *rhbA* gene has a polar effect on the expression of the other genes in the operon. As expected, the mutant is unable to produce rhizobactin 1021 (Lynch *et al.* 2001). It does, however, contain NSpd in the bound polyamine fraction (M. Dunn, unpublished). We postulate that the mutant is able to produce DAP for NSpd synthesis using HSpd synthase (Hss) with Spd as substrate. The deduced amino acid sequence of the *S. meliloti* 1021 Hss has significant similarity to the *Blastochloris viridis* Hss. The latter enzyme catalyzes the canonical Hss reaction of condensing two Put molecules to form HSpd but is also able to use Spd as a substrate, producing DAP, HSpd and Put (Krossa *et al.* 2016). The *S. meliloti* 2011 *rhbA* mutant might produce DAP for NSpd synthesis using the same side reaction of its Hss. It cannot use the DAP produced in this way for rhizobactin 1021 synthesis because of the polar effect of the insertion in *rhbA* (Lynch *et al.* 2001). A *R. tropici* CIAT 899 *hss* mutant loses the ability to synthesize both HSpd and aminobutylcadaverine, suggesting that its Hss is able to utilize Put and Cad as substrates to produce this unusual polyamine (Fujihara, Abe and Yoneyama 1995; Krossa *et al.* 2016; López-Gómez *et al.* 2016).

As much as 40% of the Earth's arable land has a pH of 5.5 or lower, which reduces crop yields and, in the case of the rhizobia-legume symbiosis, inhibits nodulation (Ferguson, Lin and Gresshoff 2013). To combat acidic stress, many bacteria use acid-inducible paralogs of the biosynthetic basic amino acid decarboxylases along with cognate polyamine/amino acid antiporters. In *E. coli*, for example, the genes for an inducible ODC (*speF*) and a Put/Orn antiporter (*potE*) are in an operon that is induced by acidic conditions. SpeF decarboxylates Orn to produce Put, a reaction that consumes a proton. The Put is exported by PotE in exchange for Orn, thus allowing a cycle of cytoplasmic proton consumption to continue (Kanjee and Houry 2013).

The *S. meliloti* ODC1 is in the same enzyme family as the *E. coli* SpeF and is encoded next to a *potE* homolog (*sma0678*). While this arrangement is indicative of a role for these genes in acid stress resistance, under aerobic conditions a Rm8530 *odc1* null

mutant grows very similarly to the wild type at pH 5.5 (Becerra-Rivera *et al.* 2018). In *S. meliloti* 1021, a significant increase in *odc1* and *potE* expression occurred under microaerobic growth at neutral pH *in vitro* but not in symbiosis (Table 2). Thus, it appears that the microaerobic induction of *odc1-potE* expression is suppressed by some factor present in root nodules. It is not known if these genes are induced under acidic growth conditions.

Excess soil salinity can severely limit legume nodulation, nitrogen fixation and yield, and polyamines produced by both symbiotic partners are implicated as being important for osmotic stress resistance (da-Silva *et al.* 2017; Dunn 2017). Polyamines made by both symbionts in nodules of salt stressed legumes may function as osmolites and stabilizers of macromolecules (Botsford and Lewis 1990; Fujihara and Yoneyama 1993; Domínguez-Ferreras *et al.* 2006; López-Gómez *et al.* 2016). In the *S. meliloti-Medicago truncatula* symbiosis, application of exogenous Spd or Spm decreases the levels of reactive oxygen species produced under salt stress (López-Gómez *et al.* 2017). In bacteroids isolated from nodules formed on bean by *R. tropici* CIAT 899 under salt stress, HSpd levels were 60% higher but Put or Spd content remained unchanged with respect to bacteroids from unstressed plants. Bean plants inoculate with a CIAT 899 *hss* null mutant had a slightly increased sensitivity to salt stress (López-Gómez *et al.* 2016).

Genetically defined but biochemically uncharacterized HSpd biosynthesis mutants of two rhizobium species have been studied. A *R. etli* CNPAF512 *hss* transposon mutant had slower than wild type growth (Braeken *et al.* 2008) while a *R. tropici* CIAT899 *hss*::Ω mutant grew poorly only under salt stress (López-Gómez *et al.* 2016). The *R. etli hss* mutant was unable to swarm (Braeken *et al.* 2008), indicating a much greater importance of HSpd for motility as opposed to growth. Motility is important for host colonization by rhizobia (Tambalo, Yost and Hynes 2015). A *S. meliloti* 2011 DABAAT (RhbA) mutant was also unable to swarm (Nogales *et al.* 2010). The motility defects of *rhbA* mutants derived from *S. meliloti* strains 2011, 1021 and GR4 are most likely due to their inability to produce the siderophore rhizobactin 1021 (Nogales *et al.* 2010; Amaya-Gómez, Hirsch and Soto 2015) because, as described above for the 2011 *rhbA* mutant, these strains are probably still able to produce NSpd (Dunn 2017).

Sinorhizobium meliloti appears to have a polyamine sensing system to regulate EPS production that is homologous to the two-component system present in *V. cholerae*. The expression of the *Vibrio* polysaccharide (*vps*) genes increases in response to the concentration of cyclic dimeric (3′→5′) GMP (c-di-GMP). The working model for the control of c-di-GMP levels in response to polyamines in *V. cholerae* involves the periplasmic protein NspS, which is in close proximity to the membrane-bound c-di-GMP phosphodiesterase (PDE) MbaA. NspS is able to bind Spm, Spd and NSpd. When NspS binds NSpd, it interacts with MbaA and decreases its PDE activity, resulting in less c-di-GMP degradation and thus higher *vps* gene expression and biofilm formation. When NspS binds Spm (or Spd, if present at a high concentration), it increases the PDE activity of MbaA, thus degrading c-di-GMP and lowering *vps* gene expression and biofilm formation (Pendergraft 2012; Cockerell *et al.* 2014; Sobe *et al.* 2017). The *S. meliloti* 1021 genes *smc00991* and *smc00992* are homologs of *nspS* and *mbaA*, respectively (Cockerell *et al.* 2014). A study of strain 1021 mutants individually lacking one of 14 different proteins for c-di-GMP metabolism showed that all of them, including an *mbaA* null mutant, were significantly swarming deficient and produced more EPS than the wild type (Wang *et al.* 2010). In *S. meliloti* 2011, however, a *mbaA* knockout mutant had a slightly increased level of biofilm formation but was not affected in motility (Schäper *et al.* 2016). We are currently characterizing the putative NspS/MbaA system in *S. meliloti* to determine its role in biofilm formation in response to specific polyamines. One hypothesis is that *S. meliloti* in the rhizosphere or rhizoplane might use such a system to control biofilm formation in response to plant excreted polyamines.

Collectively, the studies described above indicate that polyamines are required for wild type growth of symbiotic nitrogen-fixing rhizobia, especially under stress conditions. Polyamines also appear to be important in motility, EPS production and biofilm formation. Verifying the participation of polyamines like Put and Spd in these processes will require studies with mutants that completely lack the ability to make them.

Regulation of intracellular polyamine levels

Polyamine levels in cells are regulated by the balance between biosynthetic and degradative pathways, import into and export from the cell, and chemical modification. Rhizobia appear to lack genes encoding polyamine-modifying enzymes such as Spd acetyltransferase (SpdG). In addition to the biosynthetic pathways described above, rhizobia encode multiple polyamine transporters as well as possible degradative pathways for Put and Cad (Dunn 2017). There are no reports on changes in the expression of polyamine catabolic pathways under different growth conditions. Changes in the expression of genes for Arg and polyamine biosynthesis and polyamine transport in *S. meliloti* grown under different conditions or as a result of specific regulatory gene mutations are summarized in Table 2. Additional results from some of these studies are mentioned below.

An inducible arginase hydrolyzes Arg to Orn and urea in aerobically-grown cells of *S. meliloti*. Under microaerobic conditions Arg is converted to Orn by the arginine deiminase pathway (ArcA1, ArcB, ArcC) (Dunn 2017). The induction of this pathway appears to be controlled by the FixLJ master regulatory system for microaerobic gene regulation. The *S. meliloti* FixLJ also appears to be involved in the microaerobic induction of the operon containing *odc1*, *baadc* (*sma0682*) and the Put/Orn antiporter *potE* (Table 2).

In *S. meliloti* 1021 the *rhbA* and *rhbB* genes encoding the enzymes for DAP synthesis are both repressed by salt stress (Table 2), which would presumably lower both NSpd (Fig. 2) and rhizobactin 1021 production under these conditions. Hyperosmotic conditions also reduced the expression of *odc2*, encoding the key enzyme for Put production (Table 2). In strain Rm8530 under salt stress, the significant decrease in *odc2* expression correlates with the reduced amounts of Put and HSpd found in the cells (Becerra-Rivera *et al.* 2018). It is not known how *odc2* expression is regulated but several features of its promoter region are relevant: (i) a sigma 70 binding site suggests it is expressed as a housekeeping gene, consistent with its major importance in Put synthesis (ii) *odc2* and the *l/odc* genes from *S. fredii*, *R. tropici* and *R. leguminosarum* (Table 1) are divergently transcribed from a LysR-type regulator and their intergenic regions contain a conserved putative binding site for the regulator (V. A. Becerra and H. Taboada, unpublished results) (iii) the promoter region encodes two riboregulatory elements that are highly expressed in log phase rich medium cultures and in minimal medium containing the *nod* gene-inducing flavonoid luteolin, but not in bacteroids (del val *et al.* 2007, 2012; Corbino *et al.* 2005).

In contrast to the general decrease in polyamine synthesis gene expression in *S. meliloti* 1021 or Rm8530 under salt stress, a proteome analysis of *S. meliloti* 2011 showed that salt stress increased the level of CANSDC, which catalyzes the final steps

Table 2. Changes in gene or protein expression in *S. meliloti* 1021 wild type or mutants detected by transcriptomic or proteomic analysis.

	Genes or proteins differentially expressed[a]	
Strain, growth conditions and references[b]	Increased expression	Decreased expression
wt, microaerobic versus aerobic (Becker *et al.* 2004; Bobik, Meilhoc and Batur 2006)	*sma0678* (Put/Orn antiporter); *sma0680* (*odc1*); *sma0682* (*baadc*); *sma0693* (*arcA1*); *sma0595* (*arcB*); *sma0697* (*arcC*)	
fixJ or *fixK* mutant versus wt (Bobik, Meilhoc and Batur 2006)		*sma0678* (Put/Orn antiporter); *sma0680* (*odc1*); *sma0682* (*adc*); *sma0693* (*arcA1*); *sma0595* (*arcB*); *sma0697* (*arcC*)
ntrR mutant versus wt, microaerobic growth (Puskás *et al.* 2004)	*sma0680* (*odc1*); *sma0595* (*arcB*); *sma0697* (*arcC*); smb21273 (Spd/Put ABC transporter substrate binding protein)	
wt with versus without NaCl stress (Rüberg *et al.* 2003; Domínguez-Ferreras *et al.* 2006)	*smc03124* (Arg ABC transorter substrate binding protein)	SMc02983 (ODC2); SMa2400 (RhbA); SMa2402 (RhbB); SMb21273 (PotD); SMb21274 (Pot B); SMc01964 Spd/Put ABC transporter ATP-binding protein); SMc00770 (PotF)
wt with versus without sucrose-induced osmotic stress (Domínguez-Ferreras *et al.* 2006)	*smc01965* (Spd/Put ABC transporter ATP-binding protein)	SMc01964 (Spd/Put ABC transporter ATP-binding protein)
fadD mutant, swarming-inducing versus non-inducing conditions (Nogales *et al.* 2010)	*sma2402* (*rhbB*)	
wt, nutrient limited versus sufficient conditions (Djordjevic *et al.* 2003)	SMa2402 (*rhbB*)	
hfq mutant versus wt (Torres-Quesada *et al.* 2010)	*smc01652* (Spd/Put ABC transporter substrate binding protein); *smc01653* (Spd/Put ABC transporter ATP-binding protein); *smc01963* (Spd/Put ATP transporter permease); *smc01965* (Spd/Put ABC transporter ATP-binding protein); *smc01966* (Put/Spd ABC transporter substrate binding protein)	

[a]Genes and proteins are listed in italics and normal font, respectively. Abbreviations: *adc*, Arg decarboxylase; *arcA1*, Arg deiminase; *arcB*, catabolic Orn carbamoyltransferase; *arcC*, carbamate kinase; *odc1*, Orn decarboxylase 1; *odc2*, Orn decarboxylase 2; PotB, Spd/Put transporter permease; PotD, Spd/Put ABC transporter substrate binding protein; PotF, Put binding periplasmic protein; RhbA, DABA aminotransferase; RhbB, DABA decarboxylase.
[b]The 1021 wild type is denoted as wt, null mutants by the neumonic for the gene inactivated. Growth was in aerobic cultures unless specified otherwise.

in both Spd and NSpd synthesis (Fig. 2 and Table 2). Although polyamine levels were not analyzed in this study, it was proposed that the presumed increase in polyamines under salt stress might directly stabilize macromolecules (Shamselden, Nyalwidhe and Werner 2006). In *S. meliloti* Rm8530 salt stress does not change the relatively high levels of Spd and NSpd produced, but the expression level of *cansdc* has not been measured (Becerra-Rivera *et al.* 2018; M. Dunn, unpublished results).

odc2 transcription in *S. meliloti* Rm8530 increased by 50% when cells were grown under acidic conditions (minimal medium at pH 5.5), which does not correlate with the large decrease in the concentrations of Put, Spd and HSpd that occurred in the cells (Becerra-Rivera *et al.* 2018). If increased Put synthesis does in fact occur due to *odc2* upregulation under acidic conditions, the polyamine must be exported from the cell or otherwise utilized for something besides HSpd or Spd synthesis.

In animals, ODC is post-transcriptionally controlled by ubiquitin-independent degradation requiring an antizyme (AZ) and the 26S proteosome. AZ has a high affinity for ODC monomers and inhibits their dimerization, thus preventing the formation of holo-ODC. AZ binding also alters the conformation of ODC and targets it for degradation by the proteosome. AZ levels are translationally controlled by ribosomal frameshifting, which increases AZ production in response to high polyamine levels (Murakami *et al.* 1990; Zhang *et al.* 2004; Pegg 2006; Ivanov and Atkins 2007; Miller-Fleming *et al.* 2015).

AZ-like proteins in *E. coli* strongly reduce ODC activity but do not lead to its degradation (Canellakis *et al.* 1993; Panagiotidis, Huang and Canellakis 1994; Ivanov, Gesteland and Atkins 1998). In *S. ruminantium,* however, a protein initially called P22, later identified as ribosomal protein L10, is required for the degradation of its L/ODC by an ATP-dependent protease. Although the sequence of L10 is unlike that of eukaryotic AZ, it is able to increase the degradation of the ODC from mouse, while the mouse AZ also promotes degradation of the *S. ruminantium* L/ODC. The L10 protein and mouse AZ recognize the same binding site in the *S. ruminantium* L/ODC, for which amino acid residues K103, K123 and K126 are essential (Yamaguchi, Takatsuka and Kamio 2002, Yamaguchi *et al.* 2006).

L10 orthologs occur in rhizobia and it would be of great interest to determine if they are involved in polyamine regulation. The *S. meliloti* ODC2 (Becerra-Rivera *et al.* 2018) has about 40% amino acid sequence identity with the L/ODC of *S. ruminantium* but conserves only the L10 binding residue K123, while K103 and K126 are substituted by alanine and the structurally similar Arg, respectively (Yamaguchi, Takatsuka and Kamio 2002; Yamaguchi *et al.* 2006).

CONCLUSIONS AND PERSPECTIVES

Most rhizobial species appear have relatively simple polyamine biosynthetic pathways and are generally limited to producing Put and HSpd. In contrast, additional pathways present in *S.*

meliloti allow it to make NSpd and Spd. The biological rational for the extended polyamine biosynthetic ability of *S. meliloti* is not known, but is not related to any obvious characteristic like genome size or the presence of extrachromosomal replicons. The enzymes for the biosynthesis of Spd are encoded on pSymB, which contains many genes for transport and EPS synthesis. Genes required for NSpd synthesis are encoded on both pSymB and pSymA. The latter replicon encodes many functions required for symbiosis. The localization of the Spd and NSpd genes to these replicons is indicative of these polyamines having non-essential but possibly important accessory functions in free-living or symbiotically associated *S. meliloti*.

In rhizobia, we do not yet know whether polyamines cause physiological effects by acting at distinct polyamine responsive targets, such as their proposed interaction with the NspS protein in *S. meliloti*, or if they play a more general role by affecting the expression or efficiency of regulatory cascades involving global regulators like ExpR or PhoB. Neither of these propositions is mutually exclusive. Whether rhizobia have a polyamine modulon is not known, but proteome analysis could be used to reveal polyamine dependent changes in regulon expression using biosynthetic mutants grown with or without chemical complementation with specific polyamines.

We may soon have sufficient knowledge to begin modifying rhizobial polyamine metabolism so as to enhance the microsymbiont's performance during legume infection and/or in symbiosis. The special significance of polyamines in stress resistance is particularly important in this regard, since the overexpression of different stress resistance genes in rhizobia has already been shown to have positive effects on symbiosis (da-Silva *et al*. 2017).

ACKNOWLEDGEMENTS

We thank Hermenegildo Taboada (CCG-UNAM) for computational analysis of the *odc2* promoter region. Victor A. Becerra-Rivera is a doctoral student from the Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and received fellowship 574042 from CONACYT.

FUNDING

Work in the author's laboratory was partially funded by DGAPA-PAPIIT Grants IN210114 and IN206317 to M.F.D.

Conflicts of interest. The authors declare that there are no conflicts of interest.

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