Universidad CEU San Pablo
CEINDO – CEU Escuela Internacional de Doctorado

PROGRAMA en Ciencia y Tecnología de la Salud

Ensayos de rizobacterias PGPR mercurotolerantes con aplicación potencial en la recuperación de suelos contaminados por mercurio

TESIS DOCTORAL

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MADRID
2023

TESIS DOCTORAL

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REGUERO

2023

“Las cosas simples son las más extraordinarias y sólo los sabios consiguen verlas”

Paulo Coelho

Agradecimientos

Me gustaría dedicar unas pocas líneas de esta Tesis para poder expresar mi gratitud a todas aquellas personas que han hecho que este momento sea posible. El poder escribir esto deja atrás el sueño para convertirse en un hecho palpable.

En primer lugar, a mis padres y abuelos por el apoyo incansable e incondicional durante toda esta aventura. Por vuestras palabras de ánimo y el orgullo de vuestros ojos. Gracias por estar siempre ahí, por vuestra paciencia, comprensión y amor. Gracias por siempre empujarme hacia delante y levantarme en cada caída. Gracias de todo corazón.

A ti, Carol, mi compañera de viaje. La que me ha visto en mis momentos más bajos, en la frustración y el desánimo. La que siempre está para reconfortarme y darme ánimos. Gracias por estar siempre a mi lado durante los largos días y noches de luchar frente al ordenador (incluso ayuda en el laboratorio cuando ha sido necesario). Gracias por todo.

Quiero dar las gracias a mis directores de Tesis, Agustín Probanza Lobo y Pedro Antonio Jiménez Gómez, por darme la oportunidad de haber realizado este trabajo y depositar en mí su confianza. Sin vosotros no habría sido posible la culminación de este proyecto. Gracias por estar siempre a mi lado, apoyarme y animarme para superarme día a día. Sois mi gran ejemplo a seguir, tanto a nivel personal como profesional. Gracias.

A mi compañera de equipo, Marina, desde mis comienzos en el laboratorio me has ayudado y guiado para llegar hasta aquí. Muchas gracias

Igualmente, a mis compañeras, Marta y Vanesa. Sin vosotras este trabajo no habría sido posible.

A mis compañeros de departamento, Paco, Maite, Paloma, Estanis, Sergio y Maria José. Siempre habéis tenido la amabilidad de colaborar, darme vuestro apoyo y aportar vuestro conocimiento para yo crecer como investigador y persona. Sobre todo, Paco y Maite, por vuestro incansable ánimo y humor en las largas horas de laboratorio.

Gracias a la Dra. Ana Rincón, del Instituto de Ciencias Agrarias (CSIC). Quien me acogió en su equipo para realizar mi estancia. Gracias por tu apoyo y por todo lo que me quedar por aprender de ti.

Igualmente, quiero agradecerle a la Universidad por haberme permitido formarme desde la carrera a la actual culminación de esta Tesis. A todas las personas que fueron partícipes de desde su origen, en mayor o menor medida. Sin esta institución y sus componentes no hubiera llegado hasta aquí.

No me olvido de todos mis amigos, que han sufrido también mis sufrimientos y alegrías del laboratorio a lo largo de estos años.

Por último y promesa, quiero agradecer a mis profesores del colegio San Francisco de Asís, Toño, Javi Yagüe e Isabel, por apoyarme desde mis comienzos académicos a desarrollar mi carrera investigadora.

A todos os quiero agradecer el haberme acompañado hasta este momento, haberme dado ánimos, haber creer en mí y darme fuerza.

GRACIAS.

La presente Tesis, titulada: "*Ensayos de rizobacterias PGPR mercurotolerantes con aplicación potencial en la recuperación de suelos contaminados por mercurio*", se presenta como un compendio de publicaciones con la conformidad de mis directores: Dr. Agustín Probanza Lobo y Dr. Pedro Antonio Jiménez Gómez.

Se han seguido los requerimientos aprobados por el Comité de Dirección de CEINDO el 30 de noviembre de 2017. Esta tesis se ha llevado a cabo en el Departamento de Ciencias Farmacéuticas y de la Salud, dentro del Grupo de Investigación Reconocido Biotecnología microbiana ambiental (MICROAMB), con número de Registro: PC05/0720 de la Facultad de Farmacia en la universidad San Pablo CEU (Madrid, España).

Esta tesis está compuesta por las siguientes publicaciones:

1. **González D.**, Blanco C., Probanza A., Jiménez P.A., Robas M. Evaluation of the PGPR Capacity of Four Bacterial Strains and Their Mixtures, Tested on Lupinus albus var. Dorado Seedlings, for the Bioremediation of Mercury-Polluted Soils. Processes. agosto de 2021;9(8):1293. DOI: https://doi.org/10.3390/pr9081293
2. **González D.**, Robas M, Fernández V, Bárcena M, Probanza A, Jiménez PA. Comparative Metagenomic Study of Rhizospheric and Bulk Mercury-Contaminated Soils in the Mining District of Almadén. Frontiers in Microbiology. 2022;13. DOI: https://doi.org/10.3389/fmicb.2022.797444
3. **González D.**, Robas M, Probanza A, Jiménez PA. Selection of mercury-resistant PGPR strains using the BMRSI for bioremediation purposes. International Journal of Environmental Research and Public Health. 2021;18(18):9867. DOI: https://doi.org/10.3390/ijerph18189867
4. **González-Reguero D.**, Robas-Mora M, Probanza A, Jiménez PA. Evaluation of the oxidative stress alleviation in *Lupinus albus* var. orden Dorado by the inoculation of four plant growth-promoting bacteria and their mixtures in mercury-polluted soils. Frontiers in Microbiology. 2022;13. DOI: https://doi.org/10.3389/fmicb.2022.907557
5. Robas Mora M, Fernandez-Pastrana V, **González-Reguero D**, Gutierrez Oliva LL, Probanza Lobo A, Jimenez Gómez PA. Oxidative stress protection and growth promotion activity of *Pseudomonas mercuritolerans sp. nov.*, in forage plants under mercury abiotic stress conditions. Frontiers in Microbiology. 2022;13. DOI: https://doi.org/10.3389/fmicb.2022.1032901
6. *Remitido para publicación*. **González-Reguero, D.**, Robas-Mora, M., Fernández-Pastrana, V.M., Probanza, A., and Jiménez, P. A. (2023). Reduction of antibiotic resistance in the rhizosphere of Lupinus albus in mercury contaminated soil mediated by the addition of PGPB. Biology

Los datos bibliométricos de cada una de las revistas donde estos artículos se han publicado aparecen sus correspondientes capítulos.

Otra producción científica vinculada a la presente tesis:

- Robas M, Jiménez PA, **González D**, Probanza A. Bio-Mercury Remediation Suitability Index: A Novel Proposal That Compiles the PGPR Features of Bacterial Strains and Its Potential Use in Phytoremediation. International Journal of Environmental Research and Public Health. 2021;18(8):4213. DOI: https://doi.org/10.3390/ijerph18084213
- Robas M, Probanza A, **González D**, Jiménez PA. Mercury and Antibiotic Resistance Co-Selection in *Bacillus* sp. Isolates from the Almadén Mining District. International Journal of Environmental Research and Public Health. 2021;18(16):8304. DOI: https://doi.org/10.3390/ijerph18168304
- Robas Mora M, Probanza Lobo A, Jiménez Gomez PA, **Gonzalez Reguero D**. Effect of plant growth-promoting bacteria on biometrical parameters and antioxidant enzymatic activities of Lupinus albus var. Orden Dorado under mercury stress. Frontiers in Microbiology. 2022;13. DOI: https://doi.org/10.3389/fmicb.2022.891882

Patentes

- *Pseudomonas agronomica* (CECT: 30551) estimulante de la germinación y el crecimiento vegetal en condiciones de estrés abiótico. Nº solicitud: 2932806

Madrid, marzo 2023

Dr. Agustín Probanza Lobo

Dr. Pedro Antonio Jiménez Gómez

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I. Introducción

1. Aspectos generales. Antecedentes, marco general y método.

1.1 El mercurio como contaminante

El mercurio (Hg), es uno de los metales pesados con mayor toxicidad. La contaminación por este elemento constituye un grave problema medioambiental, incluso a bajas concentraciones, que afecta a todos los sistemas: suelos, agua y aire (Munthe *et al*., 2019; Mariano *et al*., 2020; Ballabio *et al*., 2021).

La mayor parte del Hg del medio ambiente se encuentra en forma de sales inorgánicas de mercurio y sales organomercúricas, con la excepción del mercurio atmosférico. La especies más prevalentes en el medio son las sales mercúricas, como $HgCl_2$, Hg(OH) y HgS. Del mismo modo, el CH_3HgCl y el CH_3HgOH son los principales compuestos organomercuriales que junto con otros compuestos orgánicos se encuentra en pequeñas fracciones (Zhang and Wong, 2007). Los compuestos organomercuriales mencionados, son compuestos derivados del metilmercurio (MeHg o $[CH^3Hg]^+$), una de las especies del Hg más peligrosas, debido por su alta capacidad de bioacumularse en los tejidos de los organismos (Munthe *et al*., 2019; Gallorini and Loizeau, 2021; Li *et al*., 2022b). De esta manera, dichas transformaciones químicas permiten al Hg difundirse por el medioambiente en sus tres estados de oxidación del Hg (Barbosa *et al*., 2001).

1.2 Fuentes de contaminación

Las fuentes emisoras de Hg de mayor relevancia son las de origen natural, antropogénicas y de reemisión (UNEP, 2018; Panagos *et al.*, 2021), siendo la atmósfera el principal destinatario que las recoge y distribuye globalmente. De esta forma, la acumulación atmosférica contribuye de forma importante al transporte y descarga del Hg a múltiples ambientes. Se calcula que, desde el inicio de la revolución industrial (AMAP/UNEP, 2013) la cantidad de Hg atmosférico global ha aumentado 10 veces y que, durante toda la era posindustrial hasta nuestros días, la cantidad de Hg acumulado en suelos y sedimentos ha aumentado de 3 a 10 veces más (Munthe *et al*., 2019).

Las principales fuentes naturales de emisión de mercurio son los volcanes, con emisiones *de novo*, así como la recirculación del Hg ya acumulado en los diferentes medios, actuando el propio medio ambiente como una fuente de reemisión. En términos cuantitativos, a nivel mundial, la cantidad total de Hg emitido a la atmósfera por fuentes naturales (volcanes, suelos, bosques, lagos y el océano) oscila entre 80 – 600 toneladas anuales (Mason *et a*l., 2012; Ballabio *et al*., 2021).

Las fuentes antropogénicas de contaminación con mercurio más importantes son las descargas urbanas, materiales agrícolas, minería, combustión y descargas industriales, que emiten de 2000 - 2200 toneladas anuales, siendo la principal fuente la quema de combustibles fósiles e incineración de desechos (Seigneur *et al*., 2004; Munthe *et al*., 2019; Ballabio *et al*., 2021).

En cuanto a las diferentes áreas geográficas, el inventario de emisiones indica que son las fuentes de Hg asiáticas las que arrojan aproximadamente el 60% del Hg antropogénico global, seguido de Latinoamérica y Europa (Jaffe *et al*., 2005; Munthe *et al*., 2019). Estas emisiones proceden fundamentalmente de la industria metalúrgica de materiales no ferrosos, siendo la principal la industria del Zn, seguido por la producción a gran escala de Au, Cu y Al (Zhang and Wong, 2007; Assessment, 2018; Munthe *et al*., 2019). La cantidad de Hg emitida a la atmósfera por estas operaciones industriales en China, constituye una tercera parte del Hg total liberado a la atmósfera por fuentes antropogénicas (Munthe *et al*., 2019).

Del mismo modo, el carbón, que es emitido a la atmósfera cuando se quema a temperaturas superiores a los 150ºC, también posee de forma natural trazas de Hg, (Finkelman, 1982; Yudovich and Ketris, 2005a, 2005b; Zhao *et al*., 2019). Así, en el carbón mineral encontramos Hg en tres formas: HgS, Hg metálico y compuestos organometálicos (Swaine, 2013; Zhao *et al*., 2019). Durante la combustión del carbón se producen una amplia cantidad de reacciones fisicoquímicas complejas que afectan a estos tres tipos de compuestos mercuriales que finalmente son emitidos a la atmósfera. La mayor parte de estas emisiones proviene de industrias que utilizan el carbón como fuente de energía, siendo las responsables de una tercera parte de las emisiones de Hg a la atmósfera (Munthe *et al*., 2019; Ishag *et al*., 2022). Así mismo, la producción de baterías y lámparas fluorescentes, cemento, minería del Hg y la quema de biocombustibles también contribuyen en buena medida a tales emisiones (Zhang and Wong, 2007; Munthe *et al*., 2019).

En los sistemas agrícolas también se emite Hg en el empleo de algunos pesticidas, fertilizantes, lodos de depuración y aguas de irrigación (Hseu *et al*., 2010).

Por último, como fuente contaminante, se encuentran las reemisiones, que se definen como las emisiones de Hg derivadas de depósitos pasados naturales y antropogénicos. Bajo las condiciones adecuadas, los depósitos de Hg en la superficie de la Tierra (suelos, rocas, nieve, hielo y agua) pueden volver a suspenderse en la atmósfera mediante mecanismos de transporte alternativos. Se estima que la reemisión anual de Hg se sitúa entre las 4.000t y las 6.300t por año (Pacyna *et al*., 2010; Mason *et al*., 2012; Munthe *et al.*, 2019; Ballabio *et al.*, 2021). La mayor parte de este mercurio reemitido acaba por acumularse de nuevo en la superficie del suelo.

1.3 Ambientes contaminados por Hg

Se conocen numerosos ecosistemas y medios contaminados con Hg, especialmente en

regiones con un alto nivel de actividad industrial y ciudades con grandes volúmenes de población (Munthe *et al.*, 2019). Los ambientes naturales en los que se detectan mayores concentraciones de Hg son los que seguidamente se indican:

1.3.1 Atmósfera

El Hg elemental (Hg^0) y el Hg divalente (Hg_2^{2+}) son las especies mayoritarias que encontramos en la atmósfera. Esta última especie está compuesta por Hg divalente gaseoso (Hg^{2+}) y partículas divalentes de Hg (Hg^p) (Engle *et al.*, 2005; Pacyna *et al.*, 2010; Munthe *et al.*, 2019; Dastoor *et al.*, 2022b).

El Hg atmosférico puede depositarse en los ecosistemas acuáticos y terrestres mediante sedimentación y lluvia. La principal especie de Hg que se deposita es el Hg divalente gaseoso, proveniente fundamentalmente de fuentes antropogénicas y de reemisión. El Hg^0 presente en la atmósfera puede depositarse en el medio tras su conversión a Hg (II) al producirse una reducción de este por el ozono (Engle *et al.*, 2005).

Recientemente, el programa del Sistema Global de Observación del Mercurio (*Global Mercury Observation System*: GMOS) de Naciones Unidas, concluye que la concentración de Hg atmosférico ha disminuido en las regiones del hemisferio norte y aumentado en el hemisferio sur. Adicionalmente se observa una tendencia a la distribución del Hg atmosférico en los países de América del norte, pudiendo encontrar los niveles más altos en las zonas altamente dependientes de la quema de carbón como fuente de energía, así como en las zonas con una elevada densidad poblacional (Munthe *et al.*, 2019). Por el contrario, la tendencia en la concentración del Hg atmosférico en Europa es a la baja. Esto puede ser debido a cada vez una menor dependencia de la quema de carbón para la producción de energía. Del mismo modo, la modernización de las plantas energéticas, las cuales incorporan sistemas de captación del Hg, ha contribuido a una reducción en las emisiones. Cabe destacar como los países del centro y del este europeo son aquellos que aportan los datos más altos de Hg atmosférico, coincidiendo a su vez estos datos con los países que tienen una mayor dependencia del uso de combustibles fósiles (Munthe *et al.*, 2019). Los datos encontrados en Asia muestran como las zonas urbanas más densamente pobladas poseen los niveles de Hg atmosférico más altos del planeta. Por el contrario, los niveles de las regiones remotas son similares a los detectados en Norteamérica y Europa (Munthe *et al.*, 2019). Este patrón se reproduce en diferentes áreas geográficas a nivel mundial. Su análisis subraya la importancia del transporte atmosférico por corrientes de aire de larga distancia. Diversos autores han observado como la cantidad de Hg acumulado en el ártico, así como los niveles atmosféricos de este, responden a dichas corrientes atmosféricas capaces de transportar el metal pesado desde el resto del mundo (Munthe *et al.*, 2019; Dastoor *et al.*, 2022a, 2022b).

1.3.2 Sistemas acuáticos

El metil-mercurio (MeHg) presenta una alta biodisponibilidad en los sistemas acuáticos. Esta alta disponibilidad se produce porque, bajo las condiciones bioquímicas apropiadas, el Hg inorgánico es convertido en MeHg por los microorganismos (Scott and Black, 2020; Gallorini and Loizeau, 2021). Es sabido que la principal fuente de biotransformación del Hg a MeHg en los sistemas acuáticos se produce en los sedimentos del lecho. Del mismo modo, diversos estudios a lo largo del tiempo demuestran que una cantidad importante de este MeHg se produce a lo largo de la columna de agua por los microorganismos (Gascón Díez *et al*., 2016; Soerensen *et al*., 2018; Gallorini and Loizeau, 2021; Li *et al*., 2022b). Este hecho facilita su acumulación en los organismos marinos, lo que puede producir una elevada concentración niveles de Hg en los mismos, incluso aunque se presente a concentraciones muy bajas de MeHg en el agua (Liu *et al*., 2020; Scott and Black, 2020; Li *et al*., 2022b). Este problema está bien descrito en determinados ambientes tales como las desembocaduras de ríos, en el paso de estos por grandes urbes y en el mar del sur de china (Liu *et al.*, 2014; Yin *et al.*, 2016; Xiang *et al.*, 2018; Bernalte *et al.*, 2020). Estudios como el de Gopikrishna *et al.* (2020) o Braune *et al.* (2015) recogen datos de cómo el Hg inorgánico en aguas del ártico canadiense.

De forma general se acepta que en torno al 95% del MeHg está biodisponible, pero a lo largo de la literatura podemos ver como este dato no está totalmente consensuado. Bradley *et al.* (2017), analiza los resultados de 20 estudios a través de los cuales se puede extraer que el dato de que la biodisponibilidad del MeHg, en productos provenientes del mar, se sitúa entre el 12 y el 79%. Del mismo modo también se encuentran discrepancias entre la disminución observada de las emisiones de Hg a la atmósfera y el Hg encontrado en los diferentes sistemas acuáticos. A pesar de esta disminución general de las emisiones y, por ello, una menor deposición atmosférica del Hg, la concentración de este en los sistemas acuáticos del mundo se ha mantenido (Munthe *et al.*, 2019; Gallorini & Loizeau, 2021). Este hecho pone de manifiesto cómo los diferentes sistemas acuáticos, y fundamentalmente los océanos, actúan como sumideros de Hg, con la consiguiente facilidad de biotransformación del Hg y su consecuente penetración en la cadena trófica.

1.3.3 Suelo

Más del 90% del Hg emitido termina de nuevo en ecosistemas terrestres, siendo el suelo el mayor depósito de este metal (Obrist, 2012; Obrist *et al.*, 2016; Ballabio *et al.*, 2021). Alrededor del 1-3% del Hg presente en los suelos es MeHg y el porcentaje restante se corresponde con diferentes complejos, manteniéndose una pequeña parte como Hg^0 (Revis *et al.*, 1990). El mercurio acumulado en suelo despierta interés debido a la fácil transmisibilidad del propio suelo a organismos que se desarrollan en él. De esta forma es capaz de incorporarse a la cadena trófica, a través de su bioacumulación en plantas para consumo humano o de ganado.

El Hg acumulado en el suelo proviene principalmente de las deposiciones atmosféricos de

larga, media y corta distancia. De esta manera los niveles más altos de Hg en suelo se encuentran cercanos a centros urbanos con una altas actividad industrial, así como, áreas mineras (Ballabio *et al.*, 2021; Panagos *et al.*, 2021).

El Hg forma, en condiciones naturales, complejos primarios con Cl^-, OH^-, S^{2-} y con compuestos orgánicos que contengan grupos funcionales azufrados. También se ha visto que los compuestos orgánicos son el factor dominante en la movilización del Hg en suelos de carácter ácido, mientras que compuestos minerales influyen más sobre la solubilización del Hg en suelos de carácter alcalino (Xu *et al.*, 2015). Estos complejos de mercurio, ya sea en forma de sales, MeHg u otros compuestos orgánicos, son fácilmente absorbibles por diversos organismos favoreciendo su transmisibilidad a la cadena trófica o desplazándose desde los depósitos en el suelo al medio acuático (Gębka *et al.*, 2020), contaminando los medios, a gran escala, llegando en última instancia al consumo humano como se puede observar en la Figura 1.

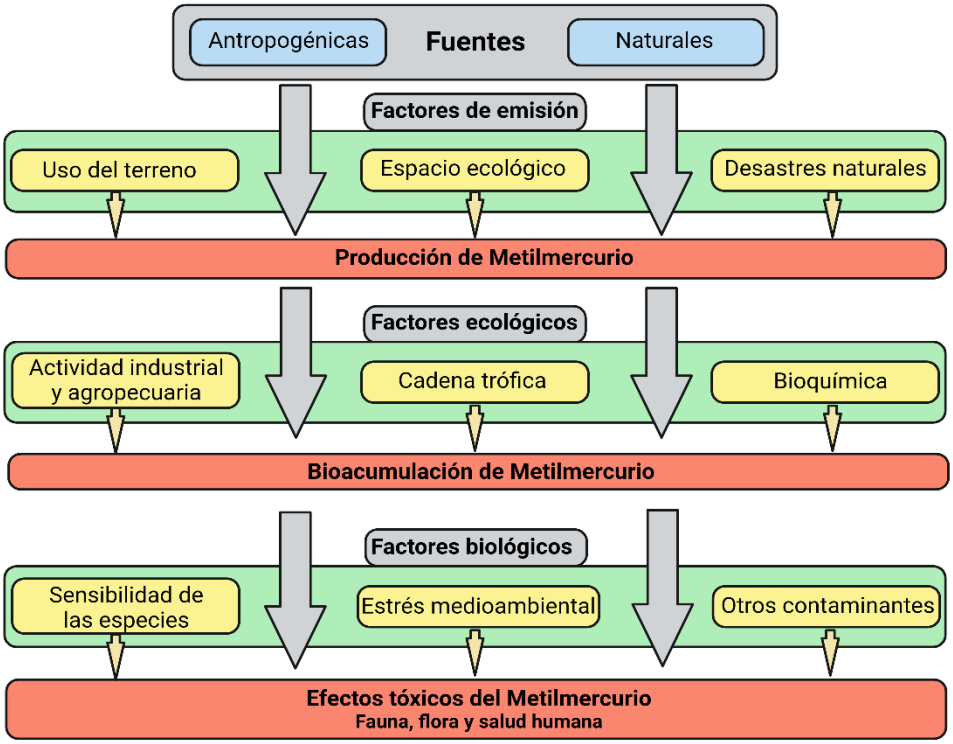

Figura 1. Flujo del Hg en el medio ambiente (Eagles-Smith *et al.*, 2016) modificado.

1.4 Efectos de la contaminación por mercurio en los sistemas y los organismos

1.4.1 Hg en cultivos y plantas

El Hg inorgánico puede incorporarse y quedar secuestrado desde los suelos a los tejidos vegetales por absorción tanto estomal como no estomal (Stamenkovic and Gustin, 2009; Arnold *et al.*, 2018; Zhou *et al.*, 2021). La asociación de este metal pesado con grupos funcionales de la materia orgánica y diversos exudados radiculares capaces de retener el Hg en el suelo

(Eagles-Smith *et al.*, 2016, 2018; Du *et al.*, 2019) favorecen la retención del Hg en los ecosistemas. Obrist *et al.*, (2016) realizó un análisis de datos sobre concentración de Hg en plantas y suelos, estimando que la retención foliar podría llegar a acumular hasta 13.000Kg (44µg m^{-2}) de Hg a lo largo de toda la región del oeste de EEUU. Adicionalmente el mercurio acumulado en la masa foliar y las fuentes de deposición húmeda de Hg, están altamente relacionadas con las concentraciones de Hg total en el suelo cuando están cercanas a plantas de producción eléctrica (que usan carbón como fuente de energía) o minas de Hg, oro o plata (Eagles-Smith *et al.*, 2016, 2018; Munthe *et al.*, 2019; Ballabio *et al.*, 2021).

La relación entre los suelos y la actividad de las plantas es uno de los marcadores ambientales más importantes de la variación del Hg inorgánico del suelo (Obrist *et al.*, 2016; Munthe *et al.*, 2019; Ballabio *et al.*, 2021). Los pronunciados gradientes de estos marcadores están profundamente influidos por la variación en las precipitaciones, mostrando que las lluvias son uno de los factores que más influyen de forma indirecta en las variaciones de concentración de Hg en el suelo. Como resultado, se observó que estas concentraciones en el suelo son, por lo general, más altas en áreas producción agrícola con una mayor acumulación de biomasa vegetal. Los datos expuestos resultan alarmantes por la alta capacidad de bioacumulación de mercurio que poseen algunos vegetales, puesto que esto puede dar lugar a una mayor facilidad para su acceso a la cadena trófica y, consecuentemente, alcanzar el consumo humano.

La concentración de Hg en cultivos de verduras y cereales cercanos a fuentes de contaminación por mercurio - entre los que encontramos minas de mercurio, plantas de producción de zinc, centrales geotérmicas, minas de carbón y zonas industriales entre otras (Chen *et al.*, 2015; Shirkhanloo *et al.*, 2015; Xu *et al.*, 2020; Wang *et al.*, 2021b) - supera el 80% en verduras y el 70% en cereales sobre los valores máximos permitidos de concentración de mercurio (Li *et al.*, 2017). Dichos estándares se sitúan en 10 µg/kg en peso fresco para los vegetales y 20 µg/kg en peso fresco de cereal, de acuerdo con los Niveles Máximos de Contaminantes en Alimentos, GB 2762-2012 (Pan *et al.*, 2016). En este mismo sentido, la Organización Mundial de la Salud (OMS) indica en sus informes que la ingesta semanal tolerable es de 1,6 µg de Hg por kilogramo de peso corporal (Li *et al.*, 2017). Considerando el peso medio mundial de un adulto de 62 kg (Walpole *et al.*, 2012), tendríamos una ingesta máxima de 62 µg /semana. Si comparamos este dato, junto con el de los niveles máximos de Hg permitidos en frutas, verduras y cereales, y con la ingesta diaria recomendada de estos productos por la Sociedad China de Nutrición (Wang *et al.*, 2016), Este dato evidencia que grandes porcentajes de la población mundial superan ampliamente el nivel máximo de consumo de mercurio semanal recomendado pudiendo llegar a una ingesta semanal de hasta 105 µg.

1.4.2 Hg en animales

Las concentraciones de mercurio que podemos encontrar en vertebrados de consumo humano son muy bajas (López-Alonso *et al.*, 2017). Sin embargo, numerosos autores recogen

evidencias de una alta concentración de Hg en pescado (Okpala *et al.*, 2018). A pesar de la gran concentración de Hg que se puede encontrar en los océanos, estudios como los realizados por Li *et al.* (2022), ponen de manifiesto como el agua de ríos y lagos posee una mayor concentración de Hg que las aguas abiertas y las costas. El Hg y el MeHg se biomagnifica a lo largo de la cadena trófica desde el fitoplancton, al zooplancton hasta organismos superiores.

El Reglamento (UE) 2018/73 de la Comisión de 16 de enero de 2018, modifican los anexos II y III del Reglamento (CE) nº 396/2005 del Parlamento Europeo y del Consejo en lo relativo a los límites máximos de residuos de compuestos de mercurio en determinados productos, DOUE 18 de enero de 2018) estipulando que la cantidad máxima permitida que pueden contener los tejidos animales de granja es de 0,01mg/kg en músculo y tejido graso y de 0,02mg/kg en vísceras. Asimismo, en esta normativa se estipula el contenido global de Hg que pueden contener los animales de caza, situándose en 0,04mg/kg. Por otra parte, el citado Reglamento hace referencia a la fuente de origen de la contaminación por mercurio en animales, poniendo de manifiesto que la contaminación medioambiental supone la amenaza principal de incorporación del Hg a la cadena trófica, hecho que contribuye a la bioacumulación en los tejidos animales destinados a consumo humano.

1.5 Hg en la cadena trófica

Los procesos conocidos como bioacumulación (acumulación de sustancias tóxicas en los tejidos) y biomagnificación (mayor acumulación de tóxicos debido a la depredación o consumo de otros organismos contaminados), afecta en última instancia, a la salud humana (Hong *et al*., 2016; Liu *et al*., 2020; Xu *et al*., 2020; Li *et al*., 2022b). La presencia de Hg en el permite su incorporación a la cadena trófica (Abeysinghe *et al*., 2017; Okpala *et al*., 2018; Li *et al*., 2022b), afectando de manera negativa, en el entorno *One health*, a la salud de los ecosistemas, especialmente a las especies que se encuentran en los niveles más altos de la cadena trófica (Gabriel and Williamson, 2004; Abeysinghe *et al*., 2017; Li *et al*., 2022b). Diversos estudios ponen de manifiesto cómo especies en posiciones altas de la cadena trófica presentan una mayor capacidad de acumulación de Hg (Li *et al*., 2022b). La mayor parte de las formas que puede adoptar el Hg en la naturaleza son altamente tóxicas para todas las especies, incluso la exposición a bajas concentraciones puede afectar al sistema nervioso central en humanos (Nance *et al.*, 2012; Gil-Hernández *et al.*, 2020; Marumoto *et al.*, 2020).

Para afrontar este problema medioambiental y de salud, la Convención de Minamata de las Naciones Unidad para la reducción de las emisiones y uso del mercurio (AMAP/UNEP, 2013). ha desarrollado regulaciones para el control de las emisiones al aire, agua, o de desechos y productos bajo estatutos Federales para el medioambiente, con las actas de *"aire limpio, agua limpia y la de recuperación y conservación de recursos naturales* (Aldy *et al.*, 2020).

1.5.1 Hg en humanos

El mercurio puede afectar en humanos a diversos sistemas y órganos, manifestándose en forma de diversas enfermedades. La exposición crónica a este contaminante, sobre todo a través del consumo de productos de origen marino, puede causar diversas alteraciones neurológicas (Eagles-Smith *et al.*, 2018; Gil-Hernández *et al.*, 2020; Marumoto *et al.*, 2020). También existen estudios que relacionan el aumento de la probabilidad de padecer enfermedades cardiovasculares con la exposición a mercurio, y en casos de exposición severa y continuada puede dar lugar a afecciones reproductivas, inmunológicas y muerte prematura (Sundseth *et al.*, 2017). Según los estudios recopilados por la OMS son dos los grupos a los que afecta el mercurio en mayor medida, los fetos y las personas que sufren una exposición mantenida en el tiempo. Un alto consumo de productos contaminados por parte de la madre durante la gestación puede dañar el cerebro y el sistema nervioso central del feto. Algunos de estos sucesos han sido analizados en regiones rurales de China asociadas al consumo de arroz contaminado (Hong *et al.*, 2016). Así mismo, también está bien analizado el caso de afección por Hg en humanos acaecido en Minamata (Japón) entre 1932 y 1968. A lo largo de ese periodo, una fábrica de ácido acético vertió líquidos residuales con alta concentración de MeHg en la bahía de Minamata, donde una gran población subsistía a base de la pesca para autoconsumo. Al menos 50.000 personas resultaron afectadas y se registraron más de 2000 casos que más tarde se conoció como el Síndrome de Minamata, alcanzando su punto álgido en 1950, con enfermos de gravedad que presentaban un cuadro de lesiones cerebrales, parálisis, habla incoherente y estados delirantes (Gil-Hernández *et al.*, 2020; Marumoto *et al.*, 2020). Aún hoy, se ha observado como en poblaciones que practican la pesca de subsistencia, entre 1,5 y 17 de cada mil niños presentan trastornos cognitivos (leve retraso mental) debido al consumo de pescado contaminado (Malagon-Rojas and Sonia, 2018).

1.5.2 Relación entre microorganismos y Hg

El mercurio como metal pesado y contaminante ejerce una fuerte presión biológica y ambiental en aquellos medios en los que se encuentra. Esta presión afecta a la estructura de las comunidades microbianas y a su diversidad (Frey and Rieder, 2013; Frossard *et al.*, 2018; Mariano *et al.*, 2020). Para sobrevivir en estos medios, las bacterias poseen diversos mecanismos para resistir las altas concentraciones de Hg en los suelos. El operón *mer*, es uno de los sistemas de defensa bacteriana más conocidos frente al mercurio (Naguib *et al.*, 2019; Bourdineaud *et al.*, 2020; Manoj *et al.*, 2020). El gen central de este operón es el gen *merA*, que codifica para un enzima mercuro-reductasa (presente en el citoplasma de la bacteria, que utiliza el NADPH como donante de electrones) cuya función es catalizar la reducción del Hg^{2+} a Hg^{0} volátil (Barkay *et al.*, 2003). Estos genes de resistencia al mercurio suelen estar incluidos en plásmidos y a otros elementos génicos móviles, ubicuos ampliamente en diversos ecosistemas. De esta manera, la proporción de bacterias con mecanismos de resistencia a Hg es directamente proporcional al nivel de contaminación por mercurio en el medio (Dash and Das,

2012).

Se conocen dos tipos de operones de operón *mer,* capaces de dotar a las bacterias de resistencia al Hg (Naguib *et al.*, 2019) (i) de espectro reducido; que confiere resistencia solo frente a Hg inorgánico; (ii) de amplio especto; además de los genes de resistencia a Hg inorgánico, incluyen genes *mer* adicionales que confieren resistencia a especies organormercúricas

El proceso bioquímico de resistencia al Hg inorgánico es muy parecido entre las diferentes especies bacterianas. En el caso de las bacterias con el operón *mer* de espectro reducido, se da una conversión del Hg^{2+} a Hg^{0} mediada por un enzima reductasa, inducida por Hg^{2+}, producida por el gen *merA*. Este enzima es una flavoproteína que utiliza el NADPH como fuente de eelctrones para la reducción del Hg^{2+} a Hg^{0}. Los iones de Hg^{2+} son transportados del entorno bacteriano al interior de la bacteria mediante el uso de diversos trasportadores transmembrana, como trasportadores ABC y RND (Qian *et al.*, 2018; Sun *et al.*, 2018; Pearson and Cowan, 2021). Como el Hg^{0} posee una presión de vapor alta, se produce la volatilización del Hg dejando el entorno bacteriano libre de este. Mientras que el proceso bioquímico de resistencia a compuestos organomercúricos, varía según la especie bacteriana con el operón *mer* de amplio espectro. La principal diferencia radica en la forma en la que el compuesto organomercúrico es transportado al citoplasma bacteriano. Tras el proceso de trasporte, el enlace entre el Hg y el carbono es digerido por una liasa, codificada en el gen *merB*, liberando Hg^{2+}. El catión de mercurio es posteriormente trasformado en Hg^{0} por una mercuro-reductasa codificada en *merA* en un proceso similar al expuesto anteriormente volatilizando el Hg (Kumari *et al.*, 2020). De esta forma, la microbiota edáfica desempeña un papel fundamental en la transformación de las diferentes especies de Hg que se pueden encontrar en el suelo.

La aparición del contaminante en los suelos propicia la selección aquellas cepas que presentan una mayor tolerancia o adquirir esta tolerancia mediante la adquisición de elementos génicos de resistencia que pueda haber en el medio (Hall *et al*., 2020; Li *et al*., 2022a). Algunos estudios (Azarbad *et al.*, 2015; Frossard *et al.*, 2017, 2018), que abarcan el estudio a corto y largo plazo de los efectos de metales pesados en los microorganismos del suelo, revelan que la tolerancia a esos metales selecciona la microbiota tolerante en unas pocas semanas o meses, pero que una completa adaptación a esta contaminación tarda años en desarrollarse. A su vez, el mercurio afecta a la composición de las comunidades bacterianas, disminuyendo su diversidad (Frossard *et al*., 2017; Mariano *et al*., 2020; Zheng *et al*., 2022).

Así mismo, esta selección natural de la tolerancia a metales pesados puede ir ligada, por un fenómeno de co-selección, a la resistencia a otros compuestos como los antibióticos. (Yan *et al*., 2020; Mazhar *et al*., 2021; Robas *et al*., 2021b), así como la transferencia horizontal de estas resistencias (Lloyd *et al*., 2016; Mahbub *et al*., 2020; Robas *et al*., 2021c; Zhao *et al*., 2021b; Li *et al*., 2022a; Zheng *et al*., 2022) Este hecho evidencia la necesidad del estudio de

zonas contaminadas con Hg.

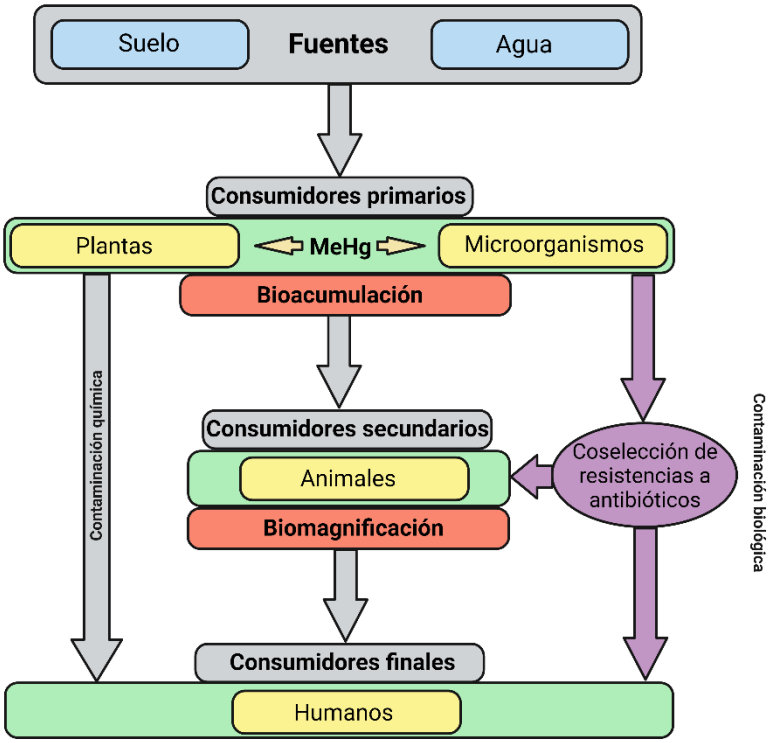

Figura 2. Flujo del Hg en la cadena trófica (contaminación química) y potencial fuente de aparición de resistencias a antibióticos (contaminación biológica). Elaboración propia.

1.6 Interacción planta-microorganismo

Las plantas y los microorganismos muestran patrones paralelos de heterogeneidad debido a que las plantas liberan una amplia gama de productos orgánicos (vía exudados), que son consumidos por los microorganismos edáficos. De esta manera, se ha postulado que la heterogeneidad taxonómica de las plantas mejora la productividad vegetal al darse un uso más eficiente a los recursos disponibles. Pero este factor ha de ir parejo con la actividad microbiana en el suelo, con especial importancia la descomposición y la biodisponibilidad de los recursos orgánicos (Goberna *et al.*, 2016).

En la interacción planta-microorganismo debemos tener en cuenta dos factores fundamentales: (i) conocer el modo en que la planta selecciona el microbiota rizosférica y el modo de relación con el microorganismo. Así conocer la composición de la rizosfera resulta de vital importancia para comprender los factores que dirigen la estructura de estas comunidades y el funcionamiento de los ecosistemas microbianos, fuertemente ligado con su contexto biótico y abiótico; (ii) comprender la diversidad funcional microbiana nos ayudará a revelar los procesos ecológicos subyacentes.

En la colaboración planta-microorganismo, el beneficio de esta sinergia en la recuperación de un suelo se ve disminuido en presencia de contaminantes (Gołębiewski *et al.*, 2014). De esta manera, se muestra como el impacto de los metales pesados altera y reduce la diversidad del ecosistema microbiano cuando estudiamos los patrones de fosfolípidos (PLFA), la biomasa o la actividad biológica de los microorganismos edáficos (Frostegård and Bååth, 1996; Mariano *et al.*, 2020).

Existen estudios previos que describen algunos aspectos referentes a la diversidad taxonómica biológica en ambientes naturales como los localizados en la zona minera de Almadén (Ruiz-Díez *et al.*, 2012), pero hay muy pocos que aborden el análisis de la heterogeneidad taxonómica global (Robas *et al*., 2021a, 2021c). Este último punto, es un aspecto de alta relevancia en la comprensión de los factores que organizan las comunidades microbianas expuestas a un contaminante. Nuestro estudio va un paso más allá de la comprensión taxonómica, pues se realiza un screening de la diversidad funcional de los microrganismos del suelo en estudio mediante el análisis metagenómico del suelo. Dicho análisis aporta una visión global del perfil funcional de los microorganismos edáficos.

A su vez, una comprensión de la diversidad taxonómica y funcional de los suelos en estudio nos ayuda a entender, en mayor medida, cómo se relacionan las comunidades con su medio y su interacción con las plantas cercanas, sobre todo las comunidades rizosféricas.

1.6.1 Selección de la microbiota por parte de la planta

Las plantas ejercen un efecto biológico y fisicoquímico sobre su entorno cercano. Los exudados de compuestos en el suelo circundante afectan directamente al suelo en el que se encuentran. Así, los exudados radiculares tienen efectos selectivos sobre las poblaciones microbianas que pueden responder con quimiotaxis o tener una respuesta de crecimiento rápido, de modo que solo un subconjunto bastante pequeño de toda la diversidad microbiana del suelo finalmente será quien colonice exitosamente las raíces (Hartmann *et al*., 2009; Huang *et al*., 2014; Vives-Peris *et al*., 2020). Por tanto, en la rizosfera se produce una interacción biológica y química muy diversa, entre las raíces de las plantas, la microbiota y las condiciones fisicoquímicas del suelo. La planta proporciona diferentes sustratos y energía hacia la rizosfera, obteniendo a cambio elementos necesarios para su desarrollo y crecimiento, como nutrientes, minerales y agua. La microbiota del suelo suele estar limitada por suministro de carbono y energía, lo que induce una secuencia compleja de actividades que también influyen en el desarrollo de las plantas.

La composición de los exudados de la raíz es crucial para la distribución del ecosistema y la especificidad del nicho de ciertas plantas (Dakora and Phillips, 2002; Hartmann *et al*., 2009; Chen *et al*., 2017; Herms *et al*., 2022). Las plantas exudan una gran variedad de compuestos orgánicos, como carbohidratos, compuestos fenólicos, sideróforos y carboxilatos para la absorción de fosfato, y antimicrobianos, que regulan la presencia potencial de microorganismos

patógenos. Del mismo modo, liberan compuestos inorgánicos que ayudan a modular pH y modula la proporción de determinadas poblaciones microbianas. Esta exudación es específica de la planta y refleja la evolución y/o la adaptación fisiológica específica a las condiciones de cada suelo (Crowley and Rengel, 1999; Cheke *et al*., 2018; Kuzyakov and Razavi, 2019). En el concepto de "rizosfera sesgada", se favorecen poblaciones microbianas específicas asociadas a la raíz en función de la modificación del perfil de exudación de la raíz (Savka *et al.*, 2013; Mohanram and Kumar, 2019; Sharma *et al*., 2021b).

Los antimicrobianos exudados tienen un efecto considerable sobre la microbiota de la rizosfera, seleccionándose sólo aquellos capaces de resistir el antibiótico producido. Por otro lado, se producen otros exudados radiculares, como azúcares, ácidos orgánicos y aminoácidos, que estimulan una respuesta quimiotáctica positiva de las bacterias (Somers *et al*., 2004; Hartmann *et al*., 2009; Yang *et al*., 2015; Jones *et al*., 2019). Ciertos compuestos como el triptófano, precursor de la producción bacteriana de ácido indol acético (IAA) promueve la colonización de los productores bacterianos de IAA. Dado que la producción de IAA rizobacteriana por bacterias asociadas a la raíz es un mecanismo importante de promoción del crecimiento de las plantas, esto tiene implicaciones importantes para el desarrollo del sistema radicular bajo la influencia de la microbiota de la rizosfera. De esta forma, la planta puede seleccionar microorganismos que puedan tener efectos específicos de promoción del crecimiento de las plantas o de biocontrol (Hartmann *et al*., 2009; Ojuederie and Babalola, 2017; Pappas *et al*., 2017; Jones *et al.*, 2019), lo que supone una gran ventaja adaptativa.

1.6.2 Bacterias promotoras del crecimiento vegetal

Durante las cuatro últimas décadas han ido prevaleciendo el uso de agentes microbiológicos (hongos y bacterias) como alternativa a los productos químicos convencionales para la producción vegetal. Las bacterias que beneficiosas para las plantas son denominadas PGPR (Plant Growth Promoting Rhizobacteria) (Kloepper, 1978) y pueden ser también empleadas como agentes biológicos estimulantes en procesos de fitorizorremediación, tanto al coadyuvar a la planta fisiológicamente en su desarrollo, como por su efecto directo sobre el contaminante con un efecto fitoprotector, o ambos efectos al mismo tiempo. En los últimos años, el término PGPR también se usa de forma indistinta como PGPB. Las especies PGPB empleadas de forma habitual son las pertenecientes los géneros *Bacillus* (Vardharajula *et al.*, 2011; Moreno-Galván *et al.*, 2020) y *Pseudomonas*. (Sandhya *et al.*, 2010). Las PGPBs estimulan el crecimiento de la planta, tanto de forma directa como indirecta.

Un efecto indirecto de la fitoprotección es la capacidad de estas bacterias para prevenir o disminuir el daño en la planta por la acción de determinados patógenos (Loon *et al.*, 1998; Kenawy *et al.*, 2019). Para ello, las bacterias acompañantes pueden producir antibióticos o inducir mecanismos de resistencia de la propia la planta. Las PGPBs pueden desarrollar otras funciones beneficiosas frente a estrés abiótico para las plantas tales como la protección frente a

la salinidad, la sequía, o compuestos tóxicos del medio como los metales pesados (Pan *et al.*, 2016; Danish *et al.*, 2020; Rajendran and Sundaram, 2020; Zerrouk *et al.*, 2020).

En cuanto a mecanismos directos de promoción del crecimiento vegetal, destacan la solubilización de fosfatos, transformando fosfatos insolubles en solubles mediante diferentes mecanismos, entre los que podemos señalar la acción de los ácidos orgánicos, quelación de los elementos responsables de la insolubilidad de los fosfatos presentes y la asimilación directa de fosfatos insolubles (Bechtaoui *et al.*, 2020) o la producción de hormonas vegetales (Khan *et al.*, 2020). La producción de ácido 3-indolacetico (AIA), la auxina más común para la estimulación de la elongación radicular (Patten y Glick, 2002; Glick, 2010; Çakmakçı *et al.*, 2020). También actúan disminuyendo los niveles de etileno en tejidos estresados mediante producción de 1-aminociclopropano-1-carboxilato desaminasa (ACCd) que degrada el precursor metabólico del etileno (Glick, 1995; Tsukanova *et al.*, 2017; Danish *et al.*, 2020; Khan *et al.*, 2020; Zerrouk *et al.*, 2020). A continuación, procedemos a describir con mayor detalle los mecanismos bacterianos más comunes de promoción directa del crecimiento vegetal:

a) Auxinas

Las auxinas son reguladores del crecimiento vegetal (RCV) responsables entre otros, de las nastias y tropismos. Estimulan procesos de elongación de las células acelerando el crecimiento de la planta; promueven la iniciación de raíces secundarias, la floración, la germinación y la correcta maduración de los frutos, entre otros procesos. Su papel en la formación de una cobertura vegetal que permita la descontaminación y protección del suelo resulta muy relevante. Por este motivo, la interacción planta-microorganismo con la participación de bacterias productoras de dicho RCV puede resultar muy beneficiosa en este tipo de procesos orientados a la recuperación de suelos contaminados (Zerrouk *et al.*, 2020).

Los metales pesados aumentan la actividad oxidativa en los vegetales (Parmar and Chanda, 2005), incluida la oxidación de ácido-3-indol- acético (AIA). Así, las plantas crecidas en presencia de Hg, incrementa el enzima AIA oxidasa en lo que inactiva el AIA. Por ello, el aporte exógeno de AIA por parte de bacterias PGPB, se considera muy relevante en la promoción vegetal en medios contaminados por este tipo de metales pesados (Chaudhry and Khan, 2007; Nedjimi, 2021; He *et al*., 2022; Xu *et al*., 2022).

b) Sideróforos

El hierro (Fe) es un elemento esencial para la mayoría de los seres vivos, siendo necesario en diversas funciones celulares tales como la síntesis de ADN, la respiración o la detoxificación de radicales libres. En la naturaleza fundamentalmente lo encontramos en la forma Fe^{3+} formando parte compuestos de baja solubilidad, lo que imposibilita su uso por algunos seres vivos. La disponibilidad de este elemento es fundamental para la colonización de un ambiente por parte de los microorganismos. Los sideróforos bacterianos actúan como agentes quelantes para secuestrar el hierro y reducirlo a Fe^{2+}, una forma mucho más soluble y aprovechable tanto en la

nutrición microbiana como vegetal (Patel and Gupta, 2020; Sarwar *et al.*, 2020).

Las plantas que son capaces de utilizar los complejos Fe^{3+}, formados por los sideróforos bacterianos, como fuente de hierro (Wang *et al.*, 1993), aumentan sus posibilidades de adaptación a diferentes condiciones de suelo. La influencia en la nutrición del hierro de los sideróforos bacterianos es altamente significativa, llegando a determinar la capacidad de supervivencia de las plantas (Wang *et al.*, 1993; Ferreira *et al.*, 2019; Schmidt *et al.*, 2020).

De este modo, las plantas poseen la capacidad de aprovechar los sideróforos microbianos. Así mismo, numerosos microorganismos en la rizosfera que pueden utilizar los fitosideróforos como quelantes para la captación de hierro. Adicionalmente, los sideróforos microbianos desempeñan un papel clave en el control de microorganismos fitopatógenos, ya sea al secuestrar el hierro, reduciendo así la disponibilidad ambiental para el crecimiento de éstos, o bien por activar los sistemas de resistencia sistémica inducida de las plantas (Arya *et al.*, 2018). Los sideróforos también son considerados fundamentales en la biorremediación de suelos contaminados por metales pesados al tener diversas funciones relacionadas con el equilibrio metálico de los suelos, así como jugar un papel fundamental en la captación de estos metales (Baldi *et al.*, 2016; Pérez-Cordero *et al.*, 2018).

c) Degradación del 1-aminocilopropano-1-carboxilato (ACC desaminasa)

La promoción del crecimiento vegetal se ve estimulada por una disminución del contenido de etileno intracelular y por la producción de amonio a partir del ACC por parte de bacterias que poseen ACC desaminasa (Glick *et al.*, 1998, 2007; Glick, 2014; del Carmen Orozco-Mosqueda *et al.*, 2020). El etileno es un RCV que se asocia al estrés vegetal y puede inhibir el crecimiento. Con ello, las cepas bacterianas PGPB con actividad ACC desaminasa son elemento de alta relevancia en la interacción planta-microorganismo (Hontzeas *et al*., 2004). Estas bacterias proliferan con mayor facilidad en el entorno rizosférico del suelo y se ven favorecidas en la competencia de nicho con otros microorganismos, lo que ha permitido emplearlas como inoculantes de plantas cultivadas en condiciones desfavorables y en presencia de contaminantes (Grobelak *et al*., 2018; del Carmen Orozco-Mosqueda *et al*., 2020; Khalifa *et al*., 2020). Además, Gontia-Mishra *et al.*, (2016) demuestran la efectividad del uso de bacterias productoras de ACC desaminasa en la promoción del crecimiento vegetal en presencia de Hg, observándose en sus estudios una mejora sustancial del crecimiento cuando bacterias de este tipo son inoculadas en cultivos, convirtiendo por este hecho a las bacterias poseedoras de esta capacidad como altamente interesantes para su uso en fitorrizorremediación.

d) Solubilización de fosfatos

La disponibilidad de fósforo es elemento crítico para el desarrollo vegetal (Rubio *et al.*, 2002). En el suelo existen diversas fuentes de fósforo que se categorizan en fósforo inorgánico y orgánico. Los factores que principalmente afectan a la distribución del fósforo en el suelo son el tipo de suelo, el pH, tipo de vegetación, actividad microbiana, además del uso de fertilizantes,

en el caso de suelos de cultivo (Rooney and Clipson, 2009; Matter *et al.*, 2020). Los microorganismos solubilizadores de fosfato son de gran importancia en la nutrición vegetal y biodisponibilidad de este recurso, ya que están involucrados en la transformación del fósforo, liberándolo desde fuentes inorgánicas mediante la solubilización de este y haciéndolo disponible desde fuentes orgánicas a través de la mineralización. Estos procesos incrementan la biodisponibilidad del fósforo del suelo, liberándolo en su forma inorgánica para ser captados por las plantas (Fankem *et al.*, 2006; Bechtaoui *et al.*, 2020; Emami *et al.*, 2020). El principal método para la solubilización de los compuestos fosfatados por las bacterias es mediante la disminución del pH de su entorno cercano a valores aproximados de pH 2. Este proceso lo llevan a cabo mediante la secreción de diferentes ácidos orgánicos como acético, láctico, málico, succínico, tartárico, glucónico, 2-cetoglucónico, oxálico y cítrico (Rodríguez *et al.*, 2006; Goswami *et al.*, 2015; Long *et al.*, 2018). Las principales rizobacterias que producen una gran cantidad de fosfatasa ácida son cepas de los géneros *Rhizobium*, *Enterobacter*, *Serratia*, *Citrobacter*, *Proteus*, *Klebsiella*, *Pseudomonas* y *Bacillus* (Chen *et al.*, 2006; Jha and Saraf, 2015; Mello *et al.*, 2019; Basu *et al.*, 2021). De esta forma, esta actividad se considera de alta importancia a la hora de fitorremediar entornos contaminados por metales pesados y por ello las bacterias capaces de solubilizar fosfatos presentes en la rizosfera de las plantas presentan un alto interés biotecnológico y son ampliamente usadas (Ahemad, 2015; Mello *et al.*, 2019).

1.7 Fitoprotección y estrés oxidativo

La fitoprotección es la capacidad protectora que pueden inducir los microorganismos a las plantas frente a agentes bióticos o abióticos que comprometan el normal desarrollo de las mismas (Mathew *et al*., 2015; Paredes-Páliz *et al*., 2018; Mello *et al*., 2019; Robas Mora *et al*., 2022). Los microorganismos fitoprotectores, frente a metales pesados, emplean fundamentalmente mecanismos de volatilización y bioadsorción (Liu *et al*., 2020b; Tiodar *et al*., 2021; Robas Mora *et al*., 2022). Esto permite que las especies vegetales se desarrollen de forma normal en ambientes contaminados. La fitoprotección es una actividad muy interesante con potencial uso agronómico en suelos contaminados que, de otra forma, tardarían largos periodos de tiempo en recuperarse. En el presente trabajo se ha analizado la capacidad fitoprotectora frente al Hg de bacterias PGPB para su empleo biotecnológico.

Algunos contaminantes, como el Hg, inducen alteraciones fisiológicas y metabólicas en las plantas, tales como la aparición de ROS (*Reactive Oxigen Species*) y disminución del crecimiento vegetal (Çavuşoğlu *et al.*, 2022). Así mismo, se conoce que el empleo de PGPB minimiza dichos efectos (Pirzadah *et al.*, 2018), estimulando diferentes mecanismos de defensa (Loix *et al.*, 2017). Uno de los principales efectos que numerosos contaminantes ambientales tienen sobre una planta, es el aumento del estrés oxidativo y la producción de ROS. El estrés oxidativo causado por el Hg ha sido estudiado en diversos modelos vegetales (Cho and Park, 2000; Cargnelutti *et al.*, 2006; Çavuşoğlu *et al.*, 2022), observándose cómo este metal pesado

aumenta el estrés y la acumulación de ROS. Así, la concentración de estos ROS es empleada como biomarcador del estrés vegetal. La producción de los enzimas superóxido dismutasa (SOD), glutatión reductasa (GR), catalasa (CAT) y ascorbato peroxidasa (APX) catalizan la degradación de ROS tales como H_20_2, HO^-, $^1O_2$ y el anión superóxido O^-_2. Por tanto, la actividad enzimática se interpreta como una respuesta protectora frente a ROS, cuya función es inducida por la presencia intracelular del Hg.

El incremento de CAT y SOD ha sido estudiado como marcador de estrés oxidativo frente a metales pesados en plantas sin inóculo bacteriano (Macar *et al.*, 2020; Çavuşoğlu *et al.*, 2022). En la presente tesis doctoral hemos podido observar cómo la actividad de estos enzimas es significativamente mayor en plantas crecidas con Hg *versus* sin Hg (González-Reguero *et al.*, 2022). Este efecto ha sido también observado por otros autores al enfrentar a las plantas a otros metales como el cadmio (Cd) o el plomo (Pb) (Sumer Aras, 2012; Azimychetabi *et al.*, 2021).

Este efecto también ha sido observado en la producción celular de los enzimas APX y GR al enfrentar diferentes especies vegetales frente a metales pesados (Hashem *et al.*, 2016; Liu *et al.*, 2018a; Azimychetabi *et al.*, 2021). Del mismo modo, Pirzadah *et al.* (2018) estudia el efecto del Hg sobre el estrés oxidativo en plantas no inoculadas con PGPB, encontrando resultados análogos a los descritos en los trabajos de la presente tesis doctoral. Del mismo modo, se conoce el efecto que la inoculación de PGPB induce en la disminución de ROS (Heidari and Golpayegani, 2012; Morcillo and Manzanera, 2021) en sustratos contaminados por diferentes metales pesados: Hg (Pirzadah *et al.*, 2018), Pb (Abdelkrim *et al.*, 2018), Cu (Fatnassi *et al.*, 2015), Zn (Islam et al., 2014a) y Cd (Azimychetabi *et al.*, 2021; Renu *et al.*, 2022).

1.8 Biorremediación

La capacidad potencial de las comunidades microbianas y su empleo para la mejora de la producción vegetal (Kloepper, 1978), así como como la utilización de la capacidad transformadora vegetal del suelo para reducir la presencia de un determinado contaminante (Eweis *et al.*, 1998; Vidali, 2001; Adams *et al.*, 2015) son cuestiones que han sido abordadas desde diferentes perspectivas. Sin embargo, la problemática de la perdida de fertilidad y diversidad de los suelos afectados por la contaminación del Hg, así como los métodos más adecuados para su recuperación, se encuentra aún en debate. La remediación de estos ambientes mediante técnicas fisicoquímicas conlleva un elevado coste y son muy agresivos con el medio ambiente (Daniel *et al.*, 2022). La biorremediación o remediación biotecnológica de un medio contaminado se presenta como una alternativa económica y con un menor impacto negativo sobre el medio ambiente. La utilización de microorganismos mercurotolerantes (Mathema *et al*., 2011), suponen una gran oportunidad para el diseño y aplicación de procesos de biorremediación eficaces (Zelles, 1999; Gadd, 2010; Saranya *et al.*, 2017; Tiodar *et al.*, 2021), ya mediante el empleo de cultivos bacterianos puros o consorcios de microorganismos,

teniendo en cuenta el efecto sinérgico con especies vegetales (Muneer *et al.*, 2013; Tiodar *et al.*, 2021).

En la literatura encontramos gran cantidad de trabajos referentes a los métodos biológicos usados en la remediación de Hg con bacterias. Robas (2017) hace una clasificación en cuatro grupos de estos trabajos, según el método de remediación utilizado:

- Volatilización del contaminante. Es sabido como bacterias que poseen el operón *mer* son capaces de reducir el mercurio de Hg^{2+} a Hg^{0} volátil (Barkay *et al.*, 2003; Dash *et al.*, 2017; Chang *et al.*, 2019; Tanwer *et al.*, 2022). Este operón tiene una alta relevancia en la captación de Hg del ambiente, su biotransformación y su reemisión. Cuenta con un sistema completo para la transformación y transporte del Hg. El operón *mer* codifica para una mercuro-reductasa (*merA*), una liasa organomercúrica (*merB*), una proteína periplásmica para la captación del Hg ambiental (*merP*), proteínas internas de membrana relacionadas con el transporte del Hg(II) (*merT*, *merC*, *merE*, *merF* y *merG*) y proteínas reguladoras del sistema y expresión del operón (*merR* y *merD*) (Gionfriddo *et al.*, 2020) .

- Biosorción de Hg. La biosorción bacteriana es definida, como la capacidad de los microorganismos para capturar metales pesados por incremento de su biomasa (Vijayaraghavan and Yun, 2008). En la biosorción bacteriana son las propias bacterias las que adsorben los contaminantes (Beveridge and Murray, 1980). El metal pesado es retenido en la pared bacteriana, sin ser necesaria la bioacumulación intracelular. Numerosos trabajos (Muneer *et al.*, 2013; Singh *et al.*, 2021; Baran *et al.*, 2022; Jing *et al.*, 2022) han estudiado bacterias y consorcios bacterianos por su capacidad de biosorción del Hg, para la remediación y eliminación de este metal pesado del medio ambiente.

- Fitoextracción (Antonkiewicz *et al.*, 2017; Zakari *et al.*, 2021; Yu *et al.*, 2022). Este es un proceso por el cual se pretende eliminar los metales pesados mediante el uso de plantas. Existen diversos vegetales que son conocidos por su capacidad bioacumuladora de metales pesados. La fitoextracción pretende aprovechar esa capacidad para la eliminación progresiva de un contaminante en el medio. En este método encontramos dos denominaciones, según la parte de la planta donde se acumule el contaminante: a) Son fitoextractoras cuando se acumula el metal en la parte aérea (Wang *et al.*, 2019), y b) son fitoestabilizadoras cuando la acumulación es en la raíz (Dary *et al.*, 2010; Ke *et al.*, 2021).

- Fitorremediación. Es una técnica que utiliza a la interacción planta microrganismo para la extracción y/o eliminación de contaminantes del suelo (Kuiper *et al.*, 2004; Raklami, 2022; Ruley *et al.*, 2022). Este método también es denominado fitorizoremediación, ya

que la interacción se da principalmente entre la planta y las bacterias presentes en su rizosfera. Esta técnica posee un efecto mucho más potente y eficiente al aprovechar la acción sinérgica entre la planta y los microorganismos. En la actualidad, la mayor parte de los trabajos van enfocados a la mejora de la capacidad fitoextractora mediante el uso de microorganismos, habiendo quedado el termino anterior englobado en la fitorremediación. Existen una gran cantidad de estudios que utilizan con éxito esta tecnica para la remediación de medios contaminados con Hg (Quiñones *et al.*, 2021; Robas *et al.*, 2021a; Tiodar *et al.*, 2021), como el que se propone en el presente trabajo.

La contaminación por Hg es sin lugar a duda uno de los problemas ambientales más importantes a los que se enfrentan aquellas zonas que, fundamentalmente por acción antropogénica debida a la explotación industrial o por la llegada de vertidos, acumulan este contaminante en el ambiente. Por ello, existe un interés científico en la búsqueda de soluciones que sirvan como paliativo a la contaminación ambiental del Hg, poniendo especial foco en aquellos lugares en los que se alcanzan concentraciones muy elevadas y evitar riesgos tanto para el medio ambiente como para la salud de los seres vivos. La biorremediación y el uso de métodos biotecnológicos para la eliminación de un contaminante del ambiente se plantea como una alternativa económica y respetuosa con el entorno. En el caso del Hg, el uso microorganismos mercurotolerantes como biorremediadores ambientales, se presenta como una gran oportunidad (Gurbanov and Severcan, 2020; Mariano *et al.*, 2020). Por tanto, el desarrollo de nuevas tecnologías que permitan una remediación ambiental efectiva es, en la actualidad, un campo de investigación de gran potencial.

1.9 Next-Generation Sequencing (NGS)

La secuenciación paralela masiva o la secuenciación paralela masiva consiste en un conjunto de técnicas de alto rendimiento para la secuenciación de ADN que utilizan el concepto de procesamiento paralelo masivo; también se denomina secuenciación de próxima generación (NGS) o secuenciación de segunda generación. Estas tecnologías emplean plataformas miniaturizadas y paralelizadas para secuenciar de 1 millón a 43 mil millones de lecturas cortas (50 a 400 bases cada uno) por cada carrera. Las plataformas NGS comparten el paradigma técnico de la secuenciación paralela masiva a través de plantillas de ADN amplificadas clonalmente separadas espacialmente o moléculas de ADN individuales en una celda de flujo. Su uso permite conocer la metagenómica de una muestra, tanto simple (células aisladas) como compleja (comunidades). La metagenómica, por tanto, consiste en el estudio completo del material genético extraído de una muestra.

Se estima que menos del 1% de las bacterias y archeas son cultivables en el laboratorio Rondon *et al.*, (2000). Para profundizar en el conocimiento de las comunidades y muestras complejas se han desarrollado diversos métodos basados en la amplificación, detección de secuencias similares de genes ya conocidos previamente o técnicas moleculares (Trajanovska

et al., 1997; Rhee *et al.*, 2004; Giagnoni *et al.*, 2018), en los que las bacterias no necesitan ser cultivadas para identificar los genes extraídos de muestras ambientales. El estudio del suelo es altamente complejo debido a su heterogeneidad, complejidad biológica en general y a la gran variabilidad microbiológica que posee. Esta complejidad y variabilidad microbiológica es una excelente oportunidad para el descubrimiento de nuevas enzimas, antibióticos y productos naturales usados por los microorganismos para competir en tales medios para ocupar el nicho ecológico. Para este fin, la biotecnología se está sirviendo de herramientas metagenómicas que aceleran los procesos de descubrimiento al analizar la matriz genética del ambiente directamente y en su totalidad, como es el objeto de este estudio. El conocimiento sobre los microorganismos, el papel que desempeñan en los ecosistemas, la diversidad taxonómica y su diversidad funcional ha sido poco profundo. Gracias a las nuevas herramientas de análisis genético se está consiguiendo poco a poco salvar ese límite de conocimiento sin necesidad de cultivo y obtener una visión generalizada en base a estos estudios.

La principal aplicación de la metagenómica, basada en la secuenciación, es la creación de librerías genéticas y reconstruir el metabolismo de los organismos que componen la comunidad y predecir sus roles funcionales en el ecosistema, mediante las llamadas “etiquetas de genes ambientales”, es decir, fragmentos de genes que codifican dominios conservados o motivos de familias de proteínas (Davenport and Tümmler, 2013; Youngblut *et al.*, 2020). Este campo también ha sido llamado genómica ambiental, ecogenómica o genómica de la comunidad (Handelsman, 2004; Riesenfeld *et al.*, 2004; Cordier *et al.*, 2021).

1.9.1 Metagenómica basada en la deteccion de amplicones

De forma tradicional y aún en la actualidad son muchos los trabajos que realizan estudios poblacionales y de diversidad flilogenética a partir del análisis del gen codificante para el *16S ARNr*. Para ellos se extrae el ADN metagenómico de la muestra de interés, aislamiento y amplificación de los 16S, lo que nos permite conocer la heterogeneidad bacteriana. Otros análisis estudian las características funcionales de las comunidades mediante el aislamiento del ARN. Con estos estudios se consigue evaluar la heterogeneidad microbiana y sus funciones en diferentes medios (Wilson and Piel, 2013; Guo *et al.*, 2016; Borah and Thakur, 2020; Hu *et al.*, 2021; Zhu *et al.*, 2022).

1.9.2 Metagenómica basada en “*Shotgun*”

Uno de los métodos de estudio más usados en metagenómica, y usado en la presente Tesis , es la técnica del *Shotgun*, mediante la cual, tras purificar el ADN de la muestra, este es cortado de forma aleatoria en muchas secuencias pequeñas que se alinean en una secuencia consenso (Zhu *et al.*, 2022). Gracias a los grandes avances en bioinformática de la última década, esta secuencia es analizada mediante programas de análisis que nos ayudan a identificar taxonómicamente y funcionalmente el ADN de la muestra.

Gracias a este corte aleatorio en fragmentos pequeños de todo el ADN de la muestra asegura que muchos de los organismos, que no serían detectados en la misma mediante las técnicas tradicionales de cultivo, aparezcan representados por lo menos en algunos fragmentos pequeños de la secuencia (Tyson *et al.*, 2004; Zhu *et al.*, 2022). Esta aproximación ha sido utilizada de forma exitosa para el estudio taxonómico y funcional de diversos suelos (Liu *et al.*, 2018b; Salam *et al.*, 2019; Enebe and Babalola, 2020; Akinola *et al.*, 2021), así como en el estudio de las resistencias a antibióticos de toda la comunidad (de Abreu *et al.*, 2020).

1.9.3 Secuenciación de genomas completos

En la última década, la mejora de los métodos de secuenciación del DNA, así como el desarrollo de nuevas técnicas, ha permitido que la obtención de genomas completos sea más rápido y accesible, introduciendo un nuevo paradigma en el estudio de los microorganismos (Didelot *et al*., 2012). La principal ventaja de estas herramientas es su universalidad, haciendo posible la obtención del genoma completo de cualquier especie microbiana sin la necesidad de su cultivo en laboratorio. Siendo posible, mediante el uso de herramientas bioinformáticas, el descubrimiento de nuevos genes (Douglas *et al*., 2020; Moon *et al*., 2020), el estudio molecular de especies incultivables (Handelsman, 2004; Cycil *et al*., 2020), así como, el descubrimiento de nuevas especies o reasignación taxonómica de estas (Mora *et al*., 2022; Robas Mora *et al*., 2022a; Shu and Huang, 2022).

1.9.4 Procesos de especiación

Se conoce como especiación al proceso mediante el cual una población de una determinada especie da lugar a otra u otras poblaciones, ya sea por aislamiento geográfico y/o adaptativo, y que mediante el acúmulo de diferencias genéticas a lo largo del tiempo irán separando más de la población de origen dando lugar a nuevas especies (Rundle and Nosil, 2005).

En los años 30 del siglo pasado, con el surgimiento de la genética moderna y fundiéndose con las hipótesis de Darwin, se comenzó el desarrollo de los modelos teórico-matemáticos con los que se formularían la Teoría Sintética de la Evolución o teoría Neodarwinista, que contempla la evolución de las especies como un proceso lento y gradual (Charlesworth *et al*., 1982; Pigliucci and Müller, 2010; Esposito, 2016; Iurato and Igamberdiev, 2022). Este proceso sería la consecuencia de la acumulación mutaciones puntuales, regulados por la acción de la selección natural y sometidos al efecto de la deriva genética y, en su caso, de las migraciones. De esta teoría surgen dos principales modos de especiación:

- Especiación alopátrida o geográfica: se produce cuando las poblaciones se ven aisladas entre sí físicamente mediante barreras geográficas que impiden el intercambio genético (Hoskin *et al*., 2005; Mukherjee *et al*., 2022). Las poblaciones aisladas entre sí, divergirán genéticamente por la aparición de nuevas mutaciones que debidos a

selección natural y deriva genética llegando a producir especies nuevas (Hoskin *et al*., 2005; Fraser *et al*., 2007).

- Especiación simpátrida: se produce cuando distintas poblaciones de una misma especie, y que ocupan un mismo territorio, se diversifican. Esto es debido a la aparición de mecanismos de aislamiento que actúan como las barreras geográficas (Bolnick and Fitzpatrick, 2007; Mukherjee *et al*., 2022). Este es el caso de la presente tesis, en el cual se da un aislamiento ecológico debido a la presencia de un contaminante y del mismo modo, otros autores describen como medios contaminados pueden ser fuente de descubrimiento de nuevos microorganismos (Morais *et al*., 2004; Lee *et al*., 2019; Cimermanova *et al*., 2021; Mora *et al*., 2022). Esto hace que distintas poblaciones se adaptan a vivir en diferentes hábitats dentro un mismo ecosistema (Friedman *et al*., 2013; Mukherjee *et al*., 2022).

1.9.5 Indicadores genéticos y umbrales para la estimación de nuevos taxones

Para la asignación genética de un organismo a una especie se ha usado tradicionalmente el gen *16S rRNA*, gen altamente conservado entre especies (Petrosino *et al*., 2009). Para ello, se ha tomado de forma general que, si el valor de identidad es superior al 98,65%, nos encontramos con organismos pertenecientes a la misma especie (Mora *et al*., 2022). Del mismo modo, si este este valor se encuentra entre el 98,65% y el 95%, se puede considerar que los organismos pertenecen al mismo género (Gwak and Rho, 2020). Sin embargo, el uso del gen *16S rRNA* para la identificación de cepas no es suficiente, dado que se conocen cepas con porcentajes de homología muy altos con respecto a especies con las que pueden diferir mucho en su fenotipo e incluso contenido génico (Gomila *et al*., 2015; Dunlap, 2019).

Por esta razón, para obtener una filogenia más robusta, recientemente se han incorporado análisis de un mayor número de genes de *housekeeping* adicionales. Aunque la comparación de estos genes es muy útil para la identificación de especies bacterianas, generalmente aisladas en el campo clínico, este marcador no permite la discriminación en muchas de las cepas ambientales aisladas (Saha *et al*., 2019).

Afortunadamente, el uso de las actuales herramientas bioinformáticas para el análisis de genoma completos, y su contenido, ha mostrado ser una herramienta útil para la discriminación y el ordenamiento taxonómico (Gutierrez-Albanchez *et al*., 2021). Con el auge de la secuenciación de *Next Generation Secuencing* (NGS), y la actual precisión y velocidad con la que se obtienen los genomas completos, hace necesario proporcionar más información para clasificar taxonómicamente las especies (Young and Gillung, 2020). La genomo-taxonomía de nuevas especies incluye el cálculo del valor de hibridación DNA-DNA (dDDH) del genoma completo con especies evolutivamente cercanas, el valor promedio de nucleótidos (por sus siglas en inglés, ANI - *Average Value of Nucleotides*) y la secuenciación completa del genoma

(Volpiano *et al*., 2021). El ANI es una medida de la similitud genómica a nivel de nucleótido entre las regiones codificantes de dos genomas (Figueras *et al*., 2014). El umbral utilizado para discriminar especies cercanas está entre 95-96% para ANI y > 70% para hibridación ADN-ADN *in silico* (dDDH) (Richter and Rosselló-Móra, 2009; Chun *et al*., 2018; Baek *et al*., 2019; Volpiano *et al*., 2021).

Del mismo modo, también se usan el índice promedio de identidad entre aminoácidos (por sus siglas en inglés, AAI - Average Amino acid Identity) y el análisis de tetra-nucleótidos (TNA). El AAI es un índice de relación genómica por pares, este índice ha mostrado tener una alta resolución en la elucidación de la estructura taxonómica más allá de la especie (Kim *et al*., 2021). Un tetranucleótido es un fragmento de secuencia de ADN con 4 bases (por ejemplo, AGTC o TTGG). Pride *et al*., (2003) demostraron que la frecuencia de tetranucleótidos en genomas bacterianos contiene marcas filogenéticas útiles para la asignación taxonómica. Aunque el análisis de tetranucleótidos (TNA) utiliza la información del genoma completo, es evidente que no puede reemplazar otros métodos filogenéticos basados en la alineación, como ANI o *16S rRNA*. Sin embargo, el TNA puede ser útil para la caracterización filogenética cuando el genoma completo o la información del gen *16S rRNA* no está disponible.

2. Aproximación conceptual de la presente tesis doctoral. Objetivos.

El suelo es uno de los recursos naturales más importantes ya que soporta a los productores primarios y a los descomponedores de materia orgánica. Así mismo, su explotación agraria y forestal contribuye al sostenimiento económico de la sociedad. Las prácticas industriales y urbanas inadecuadas lo convierten en uno de los medios receptores de la contaminación más sensibles y vulnerables. Se manifiesta, por tanto, como un sistema en equilibrio dinámico de una elevada fragilidad, susceptible de alterarse o incluso de perder dicho equilibrio natural. Por lo tanto, un uso sostenible del suelo es vital para el correcto desarrollo económico y social de las generaciones presentes y futuras. Así mismo, tanto por el impacto que la contaminación ha tenido en los últimos siglos sobre la diversidad y la salud global (*One Health*) como por la necesidad de promover un desarrollo sostenible de las sociedades modernas, la recuperación de suelos degradados se ha convertido en una prioridad de salud pública y ambiental. La presente Tesis aborda avances en el conocimiento del impacto que el Hg como contamínate ha inducido en la diversidad microbiana de las comunidades edáficas de una región expuesta a este agresor durante un periodo de más de 2000 años. De otra parte, se plantea la búsqueda de herramientas biotecnológicas que puedan postularse útiles para contribuir a la recuperación de suelos contaminados por Hg.

Para contribuir al conocimiento de los efectos del Hg sobre la diversidad de las comunidades edáficas se ha recurrido a la realización de un estudio metagenómico de amplicones del gen

completo *16S rRNA* que permita analizar la composición y diversidad taxonómico de las diferentes muestras. Asi mismo, se ha realizado un estudio de diversidad funcional empleado la técnica de Shotgun y análisis de cenoantibiograma, parámetro de síntesis desarrollado por nuestro grupo (Mora *et al*., 2017).

Para la búsqueda de nuevas herramientas biotecnológicas se ha realizado un *screening* de bacterias mercurotolerantes y se han evaluado sus capacidades PGPB en presencia de Hg, seleccionando cuatro cepas con un alto potencial biomercuroremediador (IIBMR), también desarrollado por nuestro grupo (Robas *et al*., 2021a). Dichas cepas se sometieron a ensayos de promoción del crecimiento vegetal y capacidad de fitoprotección, a fin de comprobar su idoneidad para este tipo de transformaciones. A continuación, se ensayaron bajo el modelo vegetal de *Lupinus albus* var. Orden Dorado.

Los resultados evidencian que las cepas de *Pseudomonas baetica* y *P. mercuritolerans* (así como el consorcio formado por ambas) eran capaces de inducir el crecimiento vegetal, reducir marcadores de estrés oxidativo y fitoproteger a la plata frente al Hg, disminuyendo su bioacumulación. Del mismo modo, se realizó un análisis de perfil de resistencias a antibióticos del suelo (cenoantibiograma) antes y después de la inoculación de los suelos, pudiendo comprobar como una cepa de *Brevibacterium frigoritolerans* era capaz de disminuir significativamente las concentraciones mínimas inhibitorias (de los suelos inoculados con dicha cepa) frente a los antimicrobianos.

De estos ensayos se seleccionaron las dos cepas de *Pseudomonas* para el análisis completo de su secuencia génica. Mediante su estudio genético se descartó la presencia de factores de virulencia y se pudo llegar a la conclusión de que la cepa de *P. moraviensis* se trataba de una nueva especie, siendo renombrada como *Pseudomonas mercuritolerans* (Mora *et al*., 2022).

2.1 Zona de estudio: Hg en suelo con alta concentración

En el distrito minero de Almadén (Ciudad Real), se encuentra la concentración natural de Hg más elevada del mundo (Millan *et al.*, 2011; Ballabio *et al.*, 2021; Panagos *et al.*, 2021). Nuestro grupo lleva casi una década estudiándolo, ya que es una de las regiones con mayores contracciones de Hg contaminante a nivel mundial. La situación de contaminación ambiental se debe a la explotación milenaria del Hg en la zona, data de época Romana, ya que es en este distrito minero donde se encuentra el mayor yacimiento (Díaz Puente, 2012), habiendo aportado más del 30% de la producción mundial en la historia reciente de su explotación y junto a otras dos localizaciones han producido el 50% del Hg mundial (Millan *et al.*, 2011). La explotación minera se ha llevado a cabo sin interrupciones, a lo largo de más de 2000 años, salvo las ocasionadas por eventos puntuales como inundaciones o incendios y factores externos, sobre todo por guerras.

Debido a la amplia explotación minera, se ha producido una gran acumulación de sedimentos en toda la zona, encontrando Hg en los estratos más superficiales del suelo con diferentes concentraciones. Los sedimentos constituyen un almacén natural de contaminantes, entre los que destaca el Hg. Este hecho es de alta relevancia pues puede afectar tanto a los ecosistemas como a la salud humana (Gómez *et al.*, 2007; Bose-O'Reilly *et al.*, 2010; Lominchar *et al.*, 2019).

En Almadén encontramos depósitos de Hg mineral, fundamentalmente en forma de cinabrio (HgS), y también en forma de Hg elemental (Hg^0) (Díaz Puente, 2012). Debido al gran tamaño de este distrito minero, las emisiones del mercurio que se han ido produciendo y la dispersión por los ríos, hace considerar esta área como una de las más afectadas del mundo, tanto por el origen natural del mercurio, como por su contaminación antropogénica (Millán *et al.*, 2007).

Tras el cese de la actividad minera en 2002, la zona se encuentra en una situación económica sensible. Este hecho motiva la realización de este estudio en la búsqueda de la remediación de este ambiente parar poder dar nuevos usos al terreno, puesto que en estos momentos el nivel de contaminación de los suelos impide su reutilización para fines agropecuarios o de cualquier otra índole.

No son pocos los estudios que diferentes grupos han realizado en el Distrito Minero de Almadén, y con una focalización de los mismos muy heterogénea. Estos estudios, abarcan desde perspectivas que incluyen los efectos del contaminante sobre los componentes abióticos (Millán *et al.*, 2007), otros describen las variaciones espaciales de las propiedades edáficas de la zona, o en la distribución del mercurio en ambientes acuáticos (Berzas *et al.*, 2003), hasta los efectos sobre las comunidades vegetales (Higueras *et al.*, 2004; Lominchar *et al.*, 2019) o acerca de los mecanismos de adaptación han desarrollado las distintas especies vegetales; la interrelación entre ambos tipos de componentes (bióticos y abióticos) ha sido igualmente estudiada (Millán *et al.*, 2007; Díaz Puente, 2012; Lominchar *et al.*, 2019). Por otra parte, se han realizado estudios de carácter eminentemente práctico. Sánchez *et al.* (2005) estudia el fraccionamiento del mercurio y otros elementos traza de los suelos de Almadén, mediante la aplicación secuencial de distintos procedimientos de extracción. Por su parte Gray *et al.* (2004) investiga la especiación y transformación microbiana de los residuos mineros y otros sedimentos de la zona objeto de estudio.

Sin embargo, son escasos los estudios que han abordado aspectos aplicables del aprovechamiento biotecnológico de las cepas rizosféricas de plantas capaces de crecer en estos suelos contaminados con mercurio. Según la búsqueda bibliográfica realizada, existen precedentes en la zona de estudio de trabajos que traten algunos de los aspectos básicos que se proponen en el presente trabajo, como son el aislamiento e identificación de rizobiaceas capaces de nodular plantas mercuro-resistentes (Ruiz-Díez *et al.*, 2012).

Los trabajos realizados por Millán *et al.* (2007) caracterizan la zona de estudio, aportando datos sobre las diferentes concentraciones de Hg que se pueden encontrar en el distrito minero Almadén (Figura 2). En el presente trabajo de Tesis , así como en trabajos previos del grupo (Robas *et al.*, 2021a), se utilizan los suelos de zonas (parcelas) ya caracterizados por Millán para realizar los diferentes estudios (Tabla 1). Concretamente se escogieron aquellos que contuvieran una mayor cantidad de Hg, en sus fracciones total, soluble e intercambiable. Siendo las fracciones solubles e intercambiables aquellas que afectan principalmente al medio, por su facilidad para ser biotransformadas e incorporadas a la cadena trófica.

Figura 3. Localización geográfica del distrito minero de Almadén. Localización de las parcelas experimentales descritas por Millán *et al.*, (2007a). En naranja las parcelas muestreadas en la presente Tesis. Parcela 2 (38º76'41.78" N; 4º45'53.58" W), y Parcela 6 (38º46'25,1" N; 4º51'3,9" W). (Elaboración propia a partir de Millán *et al.*, 2007a).

Tabla1. Caracterización de la concentración de Hg en los suelos de estudio (Millán *et al.*, 2007).

Soil	Hg Total (mg/Kg)	Hg Soluble (mg/Kg)	Hg Intercambiable (mg/Kg)
Parcela 6 (Suelo +Hg)	1710	0,609	7,3
Parcela 2 (Suelo -Hg)	5,03	0,0417	0,285

2.2 Metagenómica: Diversidad taxonómica y funcional microbiana

Como ya se ha indicado, por su capacidad de discriminación y su versatilidad, existe un creciente interés en el uso de técnicas metagenómicas para el estudio de muestras edáficas. En la bibliografía existen diversas referencias a la utilidad de estas técnicas para el estudio de muestras edáficas complejas (Li *et al.*, 2018; Westmann *et al.*, 2018; Castillo Villamizar *et al.*, 2019; Nelkner *et al.*, 2019). Los métodos basados en el análisis funcional de librerías de ADN

provenientes de toda la comunidad microbiana de un medio concreto (Handelsman *et al.*, 1998; Knietsch *et al.*, 2003; Sebat *et al.*, 2003; Enagbonma *et al.*, 2020), pueden ser una gran fuente para el descubrimiento de nuevos genes de resistencia a diferentes contaminantes, como los metales pesados (Garrido-Sanz *et al*., 2018; Kumar *et al*., 2018; Thomas *et al*., 2019; Sharma *et al*., 2021a; Chen *et al.*, 2022), el estudio de microorganismos incultivables (Handelsman, 2004; Cycil *et al.*, 2020; Hakim *et al.*, 2022; Wani *et al.*, 2022) y la creación de nuevas librerías genómicas (Enagbonma *et al.*, 2020).

En la presente Tesis, esta aproximación genómica mediante el estudio de amplicones nos permite entender la estructura taxonómica. El uso de shotgun, de los suelos de estudio, permite una visión global sobre el comportamiento funcional de los genes de la comunidad. El estudio nos ha facilitado identificar a nivel taxonómico como se distribuye la comunidad entre suelo libre y suelo rizosférico, observando como el factor raíz afecta a la selección de los diferentes taxones. Del mismo modo, se ha podido comprobar como diversos factores de resistencia a Hg, y otros metales pesados, están íntimamente ligados a la resistencia de diversos antibióticos. Así mismo, estos factores de co-resistencia a metales pesados y antibióticos aparecen en una mayor proporción en suelo rizosférico que en suelo libre.

2.3 Aislamiento y caracterización de cepas PGPB. Índice de Idoneidad Biomercurorremediador (IIBRM)

La búsqueda de cepas bacterianas con capacidades PGPB para la remediación de suelos contaminados con diversos elementos tóxicos, como los metales pesados y en especial con Hg ha sido objeto de numerosos trabajos científicos (Kuiper *et al*., 2004; Tiodar *et al*., 2021; Raklami, 2022; Tanwer *et al*., 2022).

Las principales características que permiten caracterizar una cepa como PGPB han sido la producción de diferentes fitohormonas y actividades promotoras del crecimiento (Gontia-Mishra *et al*., 2016; Grobelak *et al*., 2018; Bechtaoui *et al*., 2020; Pereira *et al*., 2020; Basu *et al*., 2021; Hsu *et al*., 2021). Así mismo, es necesario conocer la CMI frete al Hg para caracterizar su capacidad mercurotolerante (Mathema *et al*., 2011). Dichas actividades se encuentran citadas en el apartado “1.5.2. Bacterias promotoras del crecimiento vegetal” de la presente tesis con base en el criterio propuesto por nuestro grupo (Robas *et al*., 2021a) para el cálculo de IIBRM (Índice de Idoneidad Biomercuroremediadora) :

$$\text{IIBMR} = [\text{AIA (mg/mL)} + \text{ACC (1/0)} + \text{SID (cm)} + PO_4^{3-}\text{ (1/0)}] + [\text{CMB Hg (mg/mL)}]$$

Dónde 1= Presencia; 0 = Ausencia

Este índice evalúa el potencial biorremediador de las cepas de forma integrada y ponderada mediante la inclusión cada una de actividades PGPB y su resistencia al Hg en un algoritmo de síntesis. En el presente trabajo se ha modificado el método descrito por Robas *et al*. (2021a)

incorporando concentraciones conocidas de Hg para evaluar las diferentes capacidades PGP de las cepas seleccionadas. Hasta la fecha no se han encontrado muchos estudios en la literatura que analicen cómo el Hg afecta a estas actividades PGPB (Ravikumar *et al*., 2007; Moreno-Jiménez *et al*., 2011; Carlos *et al*., 2016), siendo en la presente Tesis la primera vez que se estudia la producción de AIA en presencia de Hg. Así mismo, no existe ningún estudio que aborde ambas perspectivas.

En los ensayos realizados en la presente tesis, se observa cómo estas actividades se ven disminuidas drásticamente al exponer a las cepas bacterianas a Hg. A su vez se pudieron seleccionar aquellas cepas que presentaron una mayor actividad incluso a altas concentraciones de Hg para su posterior estudio en los ensayos de promoción del crecimiento vegetal. De este ensayo se seleccionaron las cuatro cepas con un IIBRM más alto: *Pseudomonas mercuritolerans*, *Pseudomonas baetica*, *Brevibacterium frigoritolerans* y *Bacillus toyonensis*.

2.4 Promoción del crecimiento vegetal y fitoprotección

Es necesario comprobar las capacidades PGPB y la fitoprotección de las cepas una vez seleccionadas. Diversos estudios realizan esta aproximación tanto en sustratos inertes, ensayos de laboratorio con suelo y ensayos de campo (Mirza *et al*., 2001; Islam *et al*., 2014b; Marinkovic *et al*., 2018; Mendis *et al*., 2018; Quiñones *et al*., 2021; Robas Mora *et al*., 2022b; Yuan *et al*., 2022). En los apartados “1.5. Interacción planta-microorganismo” y “1.6. Fitoprotección y estrés oxidativo” ya se ha comentado los procesos que se utilizan como variables para la medición de las capacidades PGPB de las cepas bacterianas y de los procesos de fitoprotección que se pueden dar por parte de los microorganismos.

En este trabajo se realizó un ensayo de laboratorio con suelo proveniente del distrito minero de Almadén, a fin de poder discernir el potencial biorremediador en suelos contaminados. Obteniendo como resultado que las cepas de *P. mercuritolerans* y *P. baetica*, el consorcio formado por estás dos, así como el consorcio formado por *Pseudomonas mercuritolerans* y *Brevibacterium frigoritoleras* poseen una alta capacidad promotora del crecimiento vegetal.

Del mismo modo, se analizó el nivel de estrés de la planta mediante el análisis de los enzimas CAT, APX, SOD y GR. Se pudo observar cómo las cepas de *P. mercuritoleras*, *P. baetica* y su consorcio eran capaces de disminuir los niveles de actividad de estos enzimas, revelando un grado de estrés menor de la planta cultivada en las mismas condiciones de presencia de Hg.

Por último, se midió la acumulación de Hg en planta, observándose como las plantas inoculadas con estas cepas de *Pseudomonas* y el consorcio de ambas bioacumulaban una menor cantidad del metal pesado en sus tejidos.

2.5 Cenoantibiograma y valoración de la resistencia a antibióticos

La presencia de antibióticos en los suelos es un hecho inherente a estos, debido a que los sistemas edáficos son el hábitat de numerosas especies microbianas que los producen de forma natural. Los antibióticos pueden, a concentraciones sub-inhibitorias, presentar funciones diferentes como la activación/desactivación de factores de virulencia o la regulación de sistemas de comunicación microbiana (Liu *et al.*, 2013; Chow *et al.*, 2021; Li *et al.*, 2021; Mojsoska *et al.*, 2021). La selección positiva de mutantes de algunos microorganismos, en respuesta a un medio con presencia de antibióticos, podría favorecer un incremento en la tasa de mutación. De esta manera, los antibióticos pueden actuar como fuerza evolutiva para la producción y selección de nuevos mecanismos de resistencias (Chow *et al.*, 2021). La presencia de genes cromosómicos codificantes de mecanismos de resistencias explica, en parte, que muchas bacterias, incluso en entornos naturales libres de antibióticos, pueden portar un amplio número de genes de resistencia de forma natural (Bombaywala *et al.*, 2021; Chow *et al.*, 2021).

El efecto de las resistencia a antibióticos en las diferentes poblaciones puede deberse a diversos factores, incluido el efecto de los procesos de comunicación microbiana y/o competencia ecológica, como el *quorum-sensing/quorum-quenching* (Zhao *et al.*, 2020; Li *et al.*, 2021), así como la respuesta a factores abióticos, como la presencia de metales pesados.

Es sabido que los suelos, sometidos a una alta presión por contaminación ambiental, actúan como reservorio natural de resistencias a antibióticos (Yan *et al.*, 2020; Liao *et al.*, 2021). La resistencia a antibióticos es un problema mundial emergente que ha atraído la atención de la comunidad científica en los últimos años. Su desarrollo y evolución en el entorno clínico es evidente (Vikesland *et al.*, 2019; Kumar *et al.*, 2021). Numerosos genes que permiten la resistencia a antibióticos encontrados en bacterias patógenas han evolucionado o han sido adquiridos a partir de comunidades microbianas ambientales (Wang *et al.*, 2021a). Del mismo modo, ha sido reportada la presencia, de forma común, de bacterias multirresistentes en el medio ambiente. Este hecho sugiere la necesidad de estudiar y entender cómo el medio ambiente puede comportarse como un reservorio de mecanismos de resistencia a antibiótica. Por esta razón, la Organización Mundial de la Salud ha declarado que los genes de la RA son un nuevo contaminante debido a su prevalencia emergente y amplia distribución. Así mismo, el objetivo 2 contenido en el “Plan de Acción Mundial sobre la Resistencia a los Antimicrobianos” expone la necesidad de reforzar los conocimientos y la base científica a través de la vigilancia y la investigación. De manera particular, incide en la importancia de incrementar el conocimiento de la aparición y propagación de las resistencias a antibióticos, entre seres humanos y animales a través del medio ambiente. Así mismo, destaca la importancia de desarrollar nuevas herramientas de investigación destinadas ampliar el conocimiento en la agricultura y la acuicultura, para combatir el impacto creciente de la resistencia a los antimicrobianos (World Health Organization, 2015).

Una de estas herramientas puede ser el llamado *cenoantibiograma*, concepto definido por

nuestro grupo y que refiere al estudio fenotípico de resistencias a antibióticos de una comunidad microbiológica completa (Mora *et al*., 2017). La interpretación del cenoantibiograma no persigue caracterizar todos los diferentes mecanismos que expliquen cada una de las resistencias a antibióticos detectadas sino el comportamiento global de la comunidad microbiana edáfica frente a los antibióticos de uso más extendido. Esta técnica se propone como un posible bioindicador tanto de la evolución de la comunidad edáfica como de la comparación entre diferentes comunidades.

La evaluación y el seguimiento de una biocenosis microbiana requiere de tests para el control microbiológico ambiental. En este sentido, la aplicación del concepto cenoantibiograma, abre la posibilidad de emplear una nueva herramienta para evaluar el efecto de tratamientos de biorremediación sobre comunidades microbianas edáficas.

En la presente tesis, el estudio comparativo del cenoantibiograma antes y después de su inoculación ha permitido evaluar la capacidad de reducir/remediar el nivel de resistencias a antibióticos de un suelo altamente contaminado por efecto de la inoculación de diferentes PGPB de manera aislada y sus respectivas parejas consorciadas. El ensayo realizado ha permitido evidenciar que la introducción de las cepas del género *Pseudomonas* seleccionadas no modifica la estabilidad del suelo al mantenerse el perfil de resistencias respecto a la no inoculación. Así mismo, el uso de la cepa de *Brevibacterium frigoritolerans* por si sola, o el consorcio conformado por esta junto con la cepa de *Pseudomonas baetica*, fueron capaces de reducir los niveles de CMI de la comunidad edáfica frente a los antibióticos testados. Esto supone un hito que nos permite postula el *cenoantibiograma* como un método que contribuye a la evaluación de la estabilidad ecológica de un suelo, así como una herramienta del estudio del impacto que puede tener la introducción de una cepa bacteriana en un ecosistema.

2.6 Selección, caracterización de cepas y descripción de nuevas especies

Los entornos contaminados, ya sea de forma natural o por fuentes antropogénicas, representan una gran oportunidad para la selección de cepas bacterianas que contengan en su genoma mecanismos de resistencias a los contaminantes con los que cohabitan. La coevolución de estas especies microbianas junto con el contaminante favorece selección natural de mecanismos biológicos que les permitan evadir o minimizar su efecto toxico. Del mismo modo, la presión ejercida por el natural favorece que estas especies microbianas puedan, por presión selectiva, evolucionar para ser más competentes en su nuevo medio. Este comportamiento evolutivo permite a los espacios contaminados convertirse en nuevos nichos favorables a procesos de especiación.

El aislamiento de microorganismos de estas características persigue su empleo biotecnológico, por ejemplo, reintroduciéndolos en los medios contaminados (Muneer *et al*., 2013; Saranya *et al*., 2017; Zhao *et al*., 2021a). La reintroducción de bacterias asociadas con plantas que los

acojan y seleccionen positivamente, favorece procesos de fitorremediación (Tiodar *et al.*, 2021; Alves *et al.*, 2022). La línea de investigación del presente trabajo ha sido, en parte, ensayar este tipo de estrategias para un ulterior empleo en la descontaminación de un medio natural

Se puede encontrar en la literatura trabajos orientados a la identificación y el aislamiento de bacterias mercurotolerantes, así como su posterior aplicación en trabajos de biorremediación de medios contaminados por este metal pesado (Saranya *et al*., 2017; Mariano *et al*., 2020; Quiñones *et al*., 2021; Tiodar *et a*l., 2021; Zhao *et al*., 2021a). Esto pone de manifiesto el notable interés que despiertan este tipo de técnicas, así como la preocupación que existe para la eliminación del Hg de determinados nichos ambientales. A pesar de este interés, la dificultad de trabajar en laboratorio con mercurio ha provocado que aún se encuentren pocos trabajos de remediación en este área de estudio.

En la presente tesis se ha realizado el aislamiento, identificación y caracterización de diversas cepas bacterianas, provenientes de un suelo altamente contaminado con Hg. Tras la evaluación de sus actividades PGPB, se ensayó su capacidad fitoprotectora, tanto a nivel de reducción del estrés oxidativo, como la capacidad de reducir la bioacumulación de Hg en planta.

Una vez evidenciada su capacidad fitoprotectora se procedió a la secuenciación del genoma completo de las dos cepas de *Pseudomonas.* La anotación de genes reveló la ausencia de factores de virulencia, mecanismos transmisibles de resistencias a antibióticos y su potencial genético como PGP. Por todas estas características, ambas cepas se han postulado como buenas candidatas para el estudio de su comportamiento como PGP en campo.

Del mismo modo el estudio y comparación con las bases de datos del genoma de la cepa de *Pseudomonas moraviensis* reveló que no coincidía en el estándar para considerarla como una cepa de la especie. El análisis de su dDDH y ANI revelaron que la cepa aislada no pertenecía a ninguna especie anteriormente descrita, por loque se procedió a su clasificación como *Pseudomonas mercuritolerans*.

2.7 Objetivos

Sobre las consideraciones recogidas en los apartados anteriores, podemos concretar los objetivos de esta Tesis. Los trabajos recogidos en esta Tesis dan continuidad a la línea de investigación del grupo de investigación en biotecnología bacteriana ambiental de la Universidad San Pablo CEU. Así pues, los objetivos planteados en el diseño de la Tesis son:

1- Primer objetivo; Abordar el **estudio metagenómico de las comunidades bacterianas rizosféricas de plantas mercuroresistentes** que habitan de forma natural en Almadén, y las **compararlas con las comunidades microbianas de suelo libre**. Del mismo modo pretendemos **analizar el perfil funcional** y la expresión de **mecanismos de resistencia a los antibióticos de uso clínico y a otros compuestos tóxicos** con

objeto de contrastar resultados de estudios previos que apuntan a su co-selección en ambientes contaminados con Hg. Todo ello se concretó en la siguiente publicación:

- **González, D**., Robas, M., Fernández, V., Bárcena, M., Probanza, A., and Jiménez, P. A. (2022). Comparative Metagenomic Study of Rhizospheric and Bulk Mercury-Contaminated Soils in the Mining District of Almadén. *Frontiers in Microbiology* 13. DOI: 10.3389/fmicb.2022.797444.

2- Segundo objetivo: **Aislamiento y caracterización de cepas tolerantes a Hg con potencial uso biotecnológico para un ulterior ensayo biomercuroremediador**. Realizar un "**screening" con metodologías clásicas de las actividades PGP y CMB frente a mercurio.** El resultado de este objetivo es el siguiente artículo:

- **González, D**., Robas, M., Probanza, A., and Jiménez, P. A. (2021). Selection of mercury-resistant PGPR strains using the BMRSI for bioremediation purposes. *International Journal of Environmental Research and Public Health* 18, 9867. DOI: 10.3390/ijerph18189867

3- Tercer objetivo: Ensayo de **crecimiento de plántulas de *Lupinus albus* var. Orden Dorado** considerando la capacidad biorremediadora de **las cepas seleccionadas en trabajos previos**, bajo condiciones controladas de laboratorio. Tratamos de evidenciar la capacidad de las bacterias con mayor IIBMR de inducir mecanismos reductores del estrés oxidativo en plantas crecidas en suelos contaminados por mercurio. Son dos las publicaciones resultantes de estos estudios:

- **González, D.,** Blanco, C., Probanza, A., Jiménez, P. A., and Robas, M. (2021). Evaluation of the PGPR Capacity of Four Bacterial Strains and Their Mixtures, Tested on Lupinus albus var. Dorado Seedlings, for the Bioremediation of Mercury-Polluted Soils. Processes 9, 1293. DOI: 10.3390/pr9081293.

- **González Reguero, D**., Jimenez Gómez P. A., Robas Mora, M., and Probanza, A (2021). Evaluation of the oxidative stress alleviation in Lupinus albus var. Orden Dorado by the inoculation of Four PGPB and Their Mixtures in Mercury-Polluted Soils. Frontiers in Microbiology, 3849. DOI: 10.3389/fmicb.2022.907557

4- El cuarto objetivo: **Estudio comparado del cenoantibiograma, un novedoso concepto que asimila el perfil fenotípico de resistencia a antibióticos de la comunidad edáfica**. La prioridad de este objetivo es analizar el **posible empleo del cenoantibiograma como bioindicador de la evolución de comunidades edáficas sometida a biorremediación**, o bien su empleo para la comparación de diferentes comunidades microbianas edáficas. En el presente trabajo se trata de evidenciar el impacto que la adición de cepas bacterianas ejercer sobre el cenoantibiograma de las comunidades que las acogen. Así mismo, se trata de postular las características que

debe poseer una cepa bacteriana para que su inoculo sea eficaz e inocuo. El resultado de este objetivo está en proceso de publicación:

- **González-Reguero, D**., Robas-Mora, M., Fernández-Pastrana, V. M., Probanza, A., and Jiménez, P. A. (2023). Use of plant growth promoting bacteria (PGPB) in the rhizosphere of Lupinus albus in mercury contaminated soils: biocontrol of antibiotic resistance. *Biology*

5- Quinto objetivo. **Secuenciación del genoma completo de cepas bacterianas con IIBMR más elevado al objeto de discernir su inocuidad y conocer sus mecanismos de resistencia a Hg**. Así mismo, basado en la secuenciación completa del genoma, **asegurar su correcta identificación y/o clasificación taxonómica**. En respuesta a este objetivo se redactó el siguiente artículo:

- Robas Mora, M., Fernández-Pastrana, V.M., **Reguero, D.G**., Oliva, L.L.G., Probanza, A., and Jimenez Gómez P. A. (2022). Oxidative stress protection and growth promotion activity of Pseudomonas mercuritolerans sp. nov., in forage plants under mercury abiotic stress conditions. *Frontiers in Microbiology*, 13. DOI: 10.3389/fmicb.2022.1032901

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II. Aportaciones científicas

A continuación, se presentan las publicaciones científicas que componen la presente Tesis en el orden indicado en los Objetivos. Así mismo, se presenta a modo introductorio junto a cada publicación, un breve resumen introductorio de cada una de ellas, así como el trabajo realizado y la información bibliométrica de las publicaciones.

1. Estudio metagenómico de comunidades bacterianas en suelos contaminados por mercurio

La metagenómica es una herramienta que permite el estudio de la estructura y función de todas las secuencias de nucleótidos de los microorganismos de una muestra compleja. De manera particular, el método “Shotgun sequencing” se emplea para secuenciar cadenas de ADN de rápida expansión de manera cuasialeatoria. Sin embargo, las comunidades de los ambientes edáficos contaminados por mercurio continúan siendo aún muy desconocidos.

En el presente trabajo se realiza un estudio metagenómico de la comunidad edáfica presente en los suelos, altamente contaminados por mercurio, del distrito minero de Almadén (Ciudad Real, España). En este estudio se compara, a nivel taxonómico y funcional, los metagenomas de las comunidad edáfica de suelo libre frente a la comunidad rizosférica de plantas autóctonas. Se pudo observar la proporción de determinados taxones bacterianos seleccionados positivamente en suelo libre (como Cyanobacteria y Acidobateria) *versus* rizosfera (donde principalmente se encontraron bacterias pertenecientes a las clases Betaproteobacteria y Gammaproteobacteria). El análisis del perfil funcional pone de manifiesto cómo estos medios, sometidos a la presión de un contaminante, pueden actuar como reservorios de resistencias a antibióticos, encontrándose diversas resistencias frente a betalactámicos, cefotaximas, vancomicina y fluoroquinolonas.

En esta publicación, realicé el trabajo experimental correspondiente a la toma y preparación de la muestra y el posterior análisis de datos. La secuenciación metagenómica se externalizó con el equipo de NGS del Parque Científico de Madrid y la empresa ERA7 Bioinformatics. Escribí el primer borrador del manuscrito, sobre el que mis directores de tesis fueron trasladándome sugerencias que fui incorporando en sucesivas versiones; siendo yo el encargado del proceso de diálogo y revisión con la revista, hasta su publicación.

Factor de impacto (2021): 6.064. **Categoría:** Microbiología (Q1 34/137). **Fuente**: JCR (Journal of citation reports)

frontiers in Microbiology

ORIGINAL RESEARCH
published: 07 March 2022
doi: 10.3389/fmicb.2022.797444

Comparative Metagenomic Study of Rhizospheric and Bulk Mercury-Contaminated Soils in the Mining District of Almadén

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OPEN ACCESS

Edited by:
Wei Zhang,
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Reviewed by:
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Columbia University, United States
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Specialty section:
This article was submitted to Terrestrial Microbiology, a section of the journal Frontiers in Microbiology

Received: *18 October 2021*
Accepted: *17 January 2022*
Published: *07 March 2022*

Citation:
González D, Robas M, Fernández V, Bárcena M, Probanza A and Jiménez PA (2022) Comparative Metagenomic Study of Rhizospheric and Bulk Mercury-Contaminated Soils in the Mining District of Almadén. Front. Microbiol. 13:797444. doi: 10.3389/fmicb.2022.797444

Soil contamination by heavy metals, particularly mercury (Hg), is a problem that can seriously affect the environment, animals, and human health. Hg has the capacity to biomagnify in the food chain. That fact can lead to pathologies, of those which affect the central nervous system being the most severe. It is convenient to know the biological environmental indicators that alert of the effects of Hg contamination as well as the biological mechanisms that can help in its remediation. To contribute to this knowledge, this study conducted comparative analysis by the use of Shotgun metagenomics of the microbial communities in rhizospheric soils and bulk soil of the mining region of Almadén (Ciudad Real, Spain), one of the most affected areas by Hg in the world The sequences obtained was analyzed with MetaPhlAn2 tool and SUPER-FOCUS. The most abundant taxa in the taxonomic analysis in bulk soil were those of Actinobateria and Alphaproteobacteria. On the contrary, in the rhizospheric soil microorganisms belonging to the phylum Proteobacteria were abundant, evidencing that roots have a selective effect on the rhizospheric communities. In order to analyze possible indicators of biological contamination, a functional potential analysis was performed. The results point to a co-selection of the mechanisms of resistance to Hg and the mechanisms of resistance to antibiotics or other toxic compounds in environments contaminated by Hg. Likewise, the finding of antibiotic resistance mechanisms typical of the human clinic, such as resistance to beta-lactams and glycopeptics (vancomycin), suggests that these environments can behave as reservoirs. The sequences involved in Hg resistance (operon mer and efflux pumps) have a similar abundance in both soil types. However, the response to abiotic stress (salinity, desiccation, and contaminants) is more prevalent in rhizospheric soil. Finally, sequences involved in nitrogen fixation and metabolism and plant growth promotion (PGP genes) were identified, with higher relative abundances in rhizospheric soils. These findings can be the starting point for the targeted search for microorganisms suitable for further use in bioremediation processes in Hg-contaminated environments.

Keywords: PGPR, antibiotics, bioremediation, shotgun metagenomics, co-selection

INTRODUCTION

Mercury (Hg) is a highly toxic element that severely affects ecosystems (Hsu-Kim et al., 2018; Liu et al., 2018). It has the capacity to enter and biomagnify in the food chain and therefore affects human health even at low concentrations (Bjørklund et al., 2019). The accumulation of Hg can lead to pathologies, with those affecting the central nervous system being the most serious, such as Minamata syndrome (Gil-Hernández et al., 2020; Marumoto et al., 2020). The presence of Hg in various ecosystems is widely described. Exceptionally, environments with extremely high concentrations of this heavy metal have been described, such as those detected in the Almadén mercury mining region (> 8889 μg/g) (US Environmental Protection Agency, 2011).

The presence of Hg in soils conditions the development of organisms that inhabit it, with bacterial communities being one of the most vulnerable groups. Some of the bacterial species capable of resisting the presence of this pollutant could be suitable in processes of remediating affected soils, this is why there is a growing scientific interest in knowing the composition of these Hg-tolerant edaphic communities (Zhao et al., 2021). There are several references to the usefulness of these techniques in soil samples (Li et al., 2018; Westmann et al., 2018; Castillo Villamizar et al., 2019; Nelkner et al., 2019) and, in particular, in soils contaminated with different toxins (Garrido-Sanz et al., 2018; Kumar et al., 2018; Thomas et al., 2019).

Metagenomics consist of the complete study of genetic material extracted from a sample. Various metagenomic methods based on either DNA amplification and sequencing or DNA fragmentation and alignment are currently available (Giagnoni et al., 2018). One of the main metagenomic techniques, based on sequencing, is the creation of genetic libraries. The bioinformatic analysis of the data obtained allows us to reconstruct the metabolism of the organisms that make up the community and to predict their potential functional roles in the ecosystem through the so-called "environmental gene labels" (Youngblut et al., 2020). This field has also been called environmental genomics, ecogenomics or community genomics (Riesenfeld et al., 2004; Cordier et al., 2021). Methods based on functional analysis of DNA libraries from the entire microbial community of a particular medium can be a great source for the discovery of new genes (Handelsman, 2004; Douglas et al., 2020; Moon et al., 2020), study of unculturable microorganisms (Handelsman, 2004; Tyson et al., 2004; Cycil et al., 2020) and creation of genomic libraries (Enagbonma et al., 2020). This approach has also been used successfully in the study of antibiotic resistance in complex communities (de Abreu et al., 2020).One of the most widely used methods of study in metagenomics, and used in this work, is the "Shotgun metagenomics" technique. It consists of purifying the sample's DNA and randomly fragmenting it into many small sequences that align into consensus sequences. These sequences are processed through analysis programs that allow taxonomic and functional identification of the sample's DNA. SUPER-FOCUS is a bioinformatic tool which use the non-annotated sequences to predict potential functional activity, this allows study the whole metagenome as one unit and reveling the functional potential profile of the whole community (Chacón-Vargas et al., 2020; Collins et al., 2021).

To this end, this study aims to: (1) Find the taxonomic proportion and composition of the microbiological community of the soils of Almadén. (2) It seeks to provide an interpretation of the ecological behavior of the community, analyzing its functional potential information with SUPER-FOCUS.

MATERIALS AND METHODS

Study and Sampling Area

The samples analyzed came from the mining district of Almadén, Ciudad Real (Spain), and were collected during the spring season Specifically, the slope "S" of Cerro Buitrones was sampled from the so-called "Plot 6" (38°46′25.1″N 4°51′03.9″W), described by other authors in previous studies (Millán et al., 2007). The concentration of Hg in this plot was 1710 mg/kg total Hg, 0.609 mg/kg soluble Hg and 7.3 mg/kg exchangeable Hg. Soil samples for the metagenomic study were obtained from the rhizosphere and bulk soil, together with plants described by Robas et al. (2021a): *Rumex induratus* Boiss. and Reut., *Rumex bucephalophorus* L., *Avena sativa* L., *Medicago sativa* L. and *Vicia benghalensis* L. (Robas et al., 2021a).

Production of Soil

To obtain samples of rhizospheric soil (RS), the root of each plant specimen was gently shaken in order to remove soil fractions that were not tightly adhering to the root. The part of the soil attached to the root was then carefully separated to make up 2 g per plant. The five rhizospheric fractions were then combined into a single sample, in order to obtain10 g of soil that was homogenized for further metagenomic study. The 10 g of bulk soil (BS) was obtained in the same way, by sampling 2 g of soil near each plant, avoiding the rhizospheric fraction. Each sample was divided into 3 technical replicates for the metagenomic analysis.

Isolation of DNA

The DNA was purified by the "DNeasy Power Soil Pro Kit" (Qiagen, United States) following the manufacturer's instructions. An enzyme lysis step with lysozyme was included in order to obtain the highest and best amount of total bacterial DNA. Purified DNA was quantified using PicoGreenTM (Thermo Fisher Scientific, United States) from 40 pg. The genetic libraries were constructed using mechanical fragmentation and adaptors ligation by TruSeq (Illumina®, United States) methodology. The metagenome sequences obtained were assembled using metaSPAdes tool (Nurk et al., 2017).

Metagenomic and Bioinformatics Analysis

DNA isolated from BS and RS samples was used for metagenomic analysis. These samples were processed and sequenced with Shotgun using Illumina® MiSeq desktop using the 2 × 250 bp paired-end reagent V2 Kits (Illumina®, United States) technology with a standard quantification pattern. Bioinformatic analysis and quality control were performed using the Fast QC tool

(Andrews, 2017). Q-score was used to predict the probability of an error in base-calling. Over 75% of bases > Q30 averaged across the entire run was considered acceptable. Raw sequence reads underwent quality trimming using Trimmomatic to remove adaptor contaminants and low-quality reads (Li et al., 2010).

Taxonomic Analysis

The MetaPhlAn2 (Metagenomic Phylogenetic Analysis v2) tool was used for taxonomic analysis (Segata et al., 2012; Truong et al., 2015). This is a computational tool for profiling the composition of microbial communities from metagenomic shotgun sequencing data. MetaPhlAn relies on unique clade-specific marker genes identified from ∼17,000 reference genomes (∼13,500 bacterial and archaeal, ∼3,500 viral, and ∼110 eukaryotic) to make taxonomic predictions. It was used bowtie2 –bt2_ps "very-sensitive" preset parameters, –tax_lev "a" for prediction of all taxonomic levels, "–min_cu_len 2000" for minimum total nucleotide length for the markers in the clade, and "–stat_q 0,1" for quantile value.

Functional Analysis

The SUPER–FOCUS tool (SUbsystems Profile by databasE Reduction) was used for the functional potential analysis of the data obtained by Shotgun metagenomics. FOCUS uses a reference database to identify subsystems (predicted protein groups with similar potential function) (Silva et al., 2016). This tool reports functional annotation using CD-HIT and with the SEED database, we reduced the references of the data set (Overbeek et al., 2004; Aziz et al., 2012). SUPER-FOCUS identifies the taxonomic profile of the data and creates a database with the subsystems for predicted organism. Metagenomic data was aligned against the database using RAPSearch2 (Zhao et al., 2012). Sequences with e-values $\leq$ 1e-5, a minimum identity of 60%, and an alignment length $\geq$ 15 amino acids were retained. This database categorizes information into three levels of detail: "level I" (large functional potential groups), "level II" (families of potential functional activities) and "level III" and SEED (specific potential functional role and the protein to which the sequences belong) (Silva et al., 2016).

Statistical Analysis

For the statistical analysis, SPSS v.27.0 program (Version 27.0 IBM Corp, Armonk, NY, United States) was used. In order to evaluate the quality of the technical replicates in each soil a Pearson correlation (r) of the percent genus abundances was done. Simpson and Shannon diversity index were also calculated with the relative abundances obtained from the taxonomical analysis to assess the ecological richness between BS and RS.

RESULTS

In the metagenomic DNA extraction and sequencing of RS and BS samples, were generated a total of 15,939,287 and 15,826,564 raw reads across the three technical replicates respectively, maintaining the 98.1% (RS) and 95.3% (BS) of the sequences after QC. The sequences were aligned and analyzed in two steps, taxonomical identification and functional annotation of the sequences. Species abundance between technical replicates was highly correlated (all comparisons $r > 0.99$ with Pearson correlation test). Processing a larger number of samples would allow obtaining statistically more complete information and reducing the limitation of the results in subsequent studies. The following results are presented divide by taxonomic identification and functional potential analysis.

Taxonomic Identification

Taxonomic profile and relative abundances of the microbial community in RS and RF was analyzed using MetaPhlAn2. The identification of organisms is based on the assignment of the gene pool to a taxon. Comparing the BS and RS samples, a difference in abundance between viruses and bacteria was observed with an apparent 21 and 79% relative abundance respectively in RS and a 2 and 98% relative abundance respectively in BS.

Figure 1 shows the relative abundances of different bacterial groups. Examining the results obtained in BS, shows that the most abundant group is Actinobacteria. However, the best represented taxon in RS is Alpharoteobacteria. Acidobacteria and Cyanobacteria only appear in BS, and Betaproteobacteria and Gammaproteobacteria are only represented in RS.

Figure 2 shows the results of the relative abundances for the species taxon. Both samples seem to have a high diversity levels [Simpson's diversity (D) and Shannon's diversity (H)], being RS diversity levels (D_{RS} = 0.14, H_{RS} = 9.18) higher than BS (D_{BS} = 0.4, H_{BS} = 4.3). In RS, 73.42% of the genetic material was identified, leaving 26.58% unidentified (**Figure 2** and **Supplementary Table 1**). Similarly, in BS 38.87% were identified. 61.13% of this DNA belongs to the various taxa (**Figure 2** and **Supplementary Table 1**).

The relative abundance of *Kribbella* sp. stands out in both samples, having a greater representation in RS. Similarly, the high representation of *Pseudomonas* sp. and *Mesorhizobium* sp. in RS (**Figure 2**) stands out. Likewise, the presence of strains of the genera *Actinoplanes* sp., *Microcoleus* sp. and *Propionibacterium* sp. seems to be especially abundant in BS (**Figure 2**).

Viral DNAs have been identified as *Cyclovirus NGChicken15/NGA/2009*, with 18.88% representation in RS. Less abundant in both samples was the mosaic Dasheen virus.

Potential Functional Analysis

The functional analysis with SUPER-FOCUS present three levels (I-III) and predicted proteins (SEED) with the non-annotated metagenome sequences that allows pool the sequences by potential functional activity clusters. The two metagenomes obtained were assembled using MetaSpades. A total of 813,375 and 676,195 contigs were obtained, respectively.

Functional Level I: Large Functional Potential Groups

In the analysis of level I (more general group of potential functional activity), the sequences pooled on the same potential functional activity were ordered according to their relative abundance. In this way, the functional potential content of

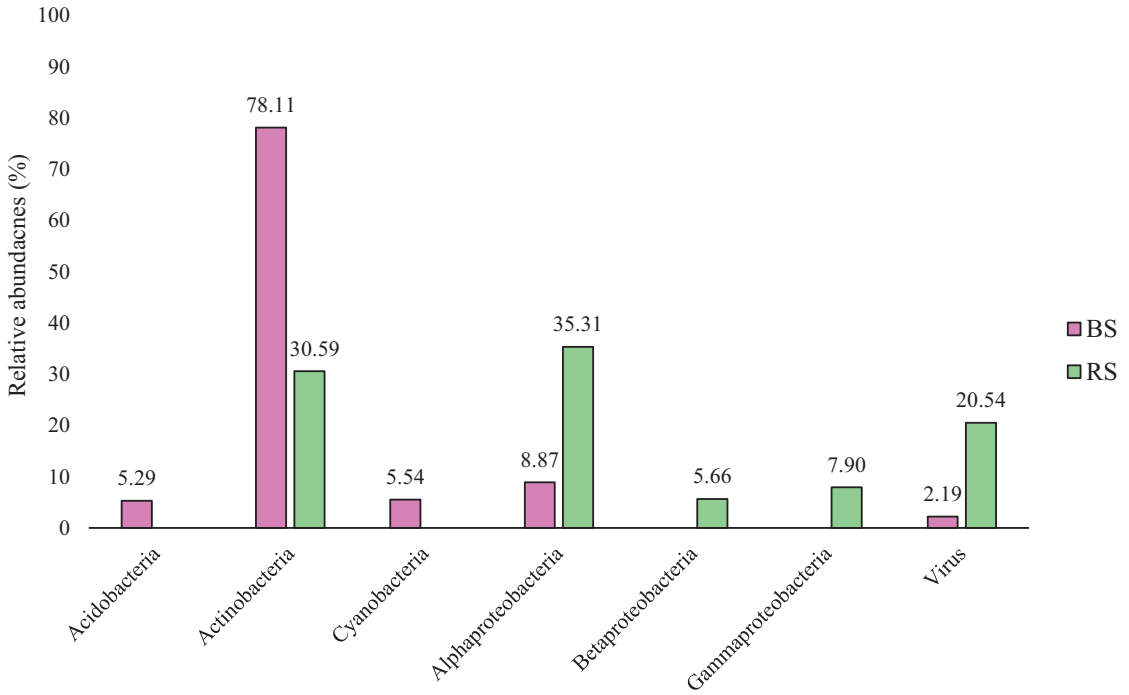

FIGURE 1 | Comparative of the Relative abundances by taxonomic level of Class in BS (Pink - Bulk Soil) and RS (Green - Rhizospheric Soil). Data in the figure show how the relative abundances of the diferent classes are further grouped according to their presence in RS or BS.

the samples was ranked, reflecting the abundance of the different subsystems.

Were found 35 functional potential groups (**Supplementary Table 2**). The most abundant subsystems are those which seem to be related to basal metabolism and basic functions for the survival of bacteria, such as the carbon cycle, amino acid synthesis, and functional activities involved in breathing, among others, while subsystems encompassing more specific characteristics (such as virulence or photosynthesis) are less represented.

It is interesting that the functional potential cluster endowment of "stress response" presents 4.45% RS and 4.36% BS abundances. This functional potential group is among the 10 most abundant, which indicates the high environmental pressure suffered by bacteria in soils contaminated with Hg.

Functional Level II: Families of Potential Functional Activities

At this level (**Figure 3**), the potential functional activities were collected in a more concrete way, ordering them according to their relative abundances by families of same potential functional activities. Were found at this level 192 functional potential clusters (**Supplementary Table 3**).

Some potential functional activities are especially relevant and could allow for explaining the biological behavior of the Hg-tolerant edaphic communities. The following activities stand out (**Figure 3**): "Resistance to antibiotics and toxic compounds" (3.03% RS and 2.96% BS), "ABC type conveyors" (1.88% RS and 1.85% BS) and "oxidative stress" (1.26% RS and 1.13% BS).

Functional Level III and SEED: Specific Potential Functional Role and the Protein to Which Sequences Belong

The third level identifies the potential functional role to which the sequences under study belong, revealing the potential function within the metabolism of the bacteria. On level III, 1,186 functional potential groups were found and 24,441 functional potential annotations on SEED were done (**Supplementary Tables 4, 5**).

The 35 most abundant subsystems belong to a division of the subfamilies found in levels I and II. Furthermore, most of the subfamilies of biological interest in this study seems to be represented with lower relative abundance percentages.

At this level, there are several subsystems that group the resistance to heavy metals and to Hg, in particular. Among them we find various predicted proteins of the *mer* operon, Hg-reductases and ABC-type transporters associated with resistance to heavy metals.

Noteworthy is the large number of subsystems could be linked to resistance to various antibiotics, including beta-lactamases, predicted proteins regulating the BlaR1 family, various proteins of multiple resistance systems, multiple resistance systems linked to the MexAB-OprM and MexEF-OprN complex, fluoroquinolone resistance, vancomycin resistance, mdtABCD flow pump cluster and bacitracin stress response.

There are also subsystems involved in the biological cycle of nitrogen (N). Particularly important are those linked to the nitrogenase complex for atmospheric N fixation. Likewise, the potential functional activities responsible for the

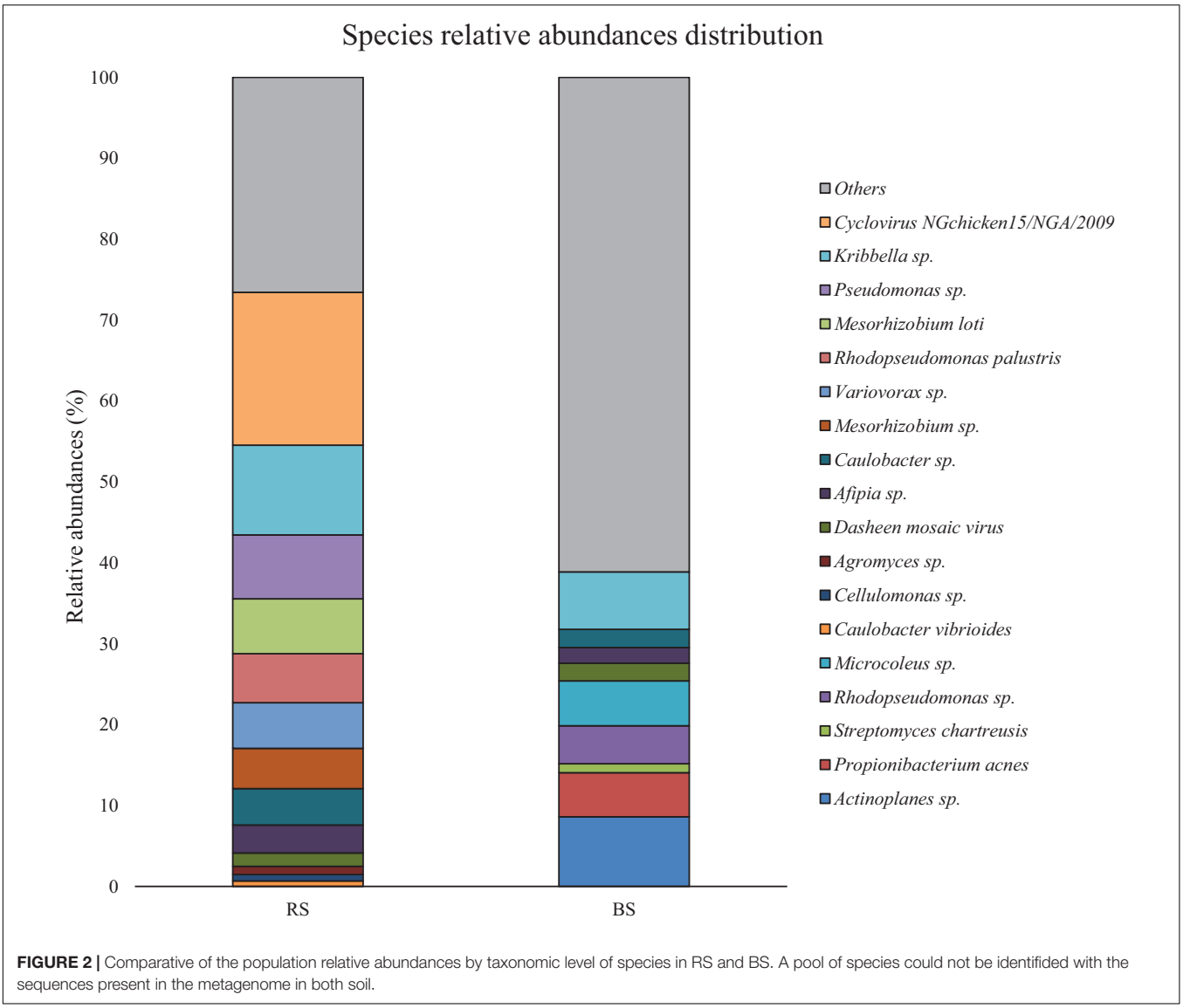

FIGURE 2 | Comparative of the population relative abundances by taxonomic level of species in RS and BS. A pool of species could not be identified with the sequences present in the metagenome in both soil.

promotion of plant growth are represented as "production of auxins," "metabolism of ethylene," "siderophore production," and "phosphate solubilization." With a major relative abundance in RS.

DISCUSSION

Taxonomic Discussion

In the soils analyzed, representatives of six taxonomic phylum appear: Acidobacteria, Actinobacteria, Cyanobacteria, Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria, whose analysis and discussion of the findings are described below. The results obtained show how the taxonomical diversity varies between bulk soil and rhizospheric soil, being positive selected by the plant root those that have a potential benefit to the plant, like proteobacteria group (**Figure 1** and **Table 1**) (Moulin et al., 2001; Zai et al., 2021).

Acidobacteria

Acidobacteria are a phylum within the bacteria domain of species ubiquitous in the soil (Kalam et al., 2020). In the present study, the sample was identified up to the family taxon Acidobacteriaceae (**Table 1**) (Foesel et al., 2016). In the same way as in our results, the predilection of this bacterial group for the bulk soil has also been described in comparison with the rhizosphere (Kielak et al., 2016; Conradie and Jacobs, 2020). Several authors have also discussed the potential of this bacterial class for the degradation of various pollutants (Feng et al., 2021; Gonçalves and Santana, 2021) and its potential biotechnological application in Hg degradation (Vishnivetskaya et al., 2011; McDaniel et al., 2020).

Actinobacteria

Actinobacteria are a phylum and class of Gram-positive bacteria and are the group with the highest representation in BS and the second most represented in RS. This taxon is particularly

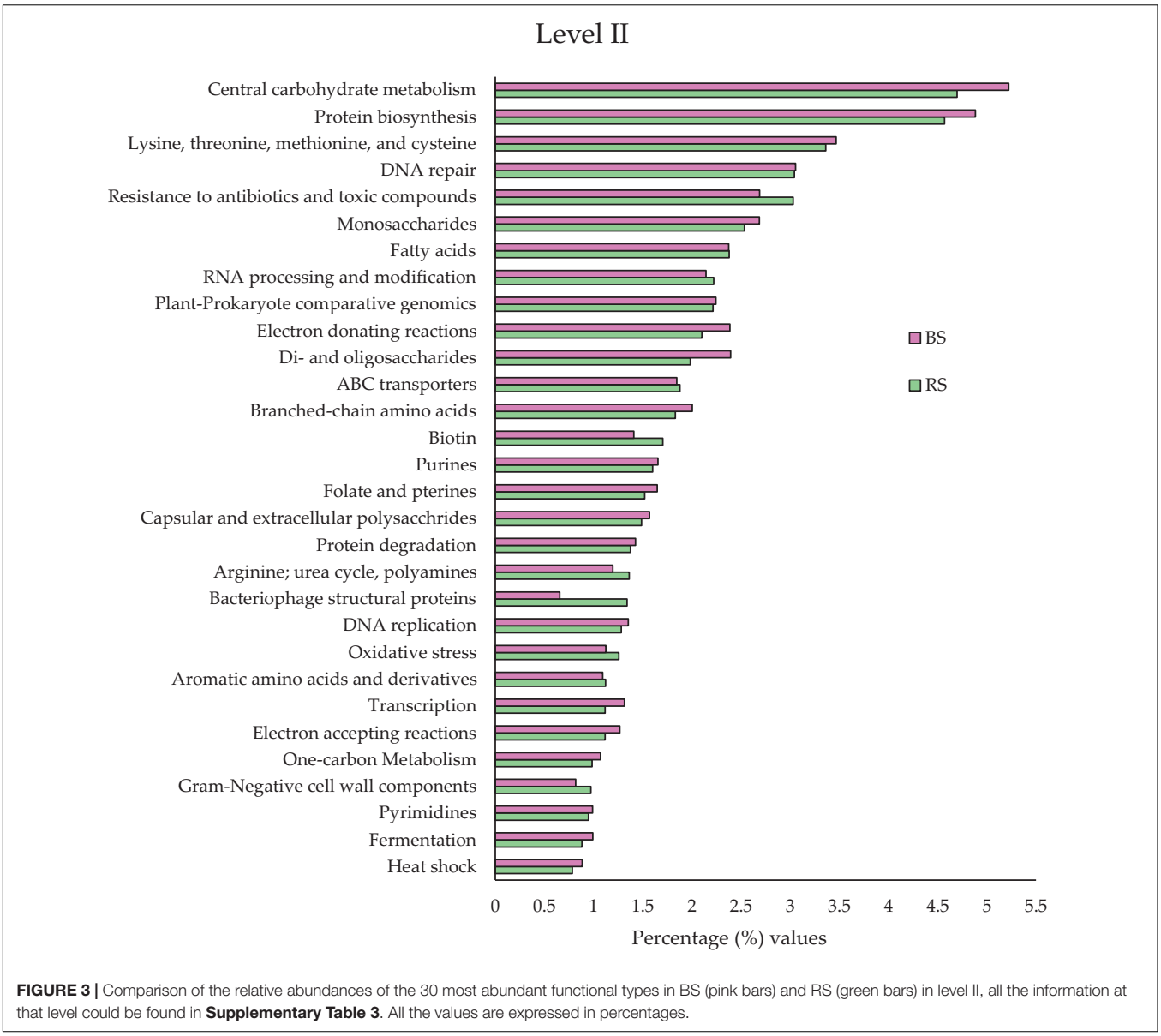

FIGURE 3 | Comparison of the relative abundances of the 30 most abundant functional types in BS (pink bars) and RS (green bars) in level II, all the information at that level could be found in **Supplementary Table 3**. All the values are expressed in percentages.

interesting because of its wide biotechnological potential. Most antibiotics and many of the compounds used in the production of medicines come from the species in this class (Hopwood, 2007). There are recent studies of the relationship of these bacteria with resistance to various heavy substances (Yun et al., 2020), among which are the mer genes of resistance to Hg (Christakis et al., 2021). In this manuscript, representatives have been found in seven of the orders pertaining to this class (**Table 1**).

Two families and two species of the Propionibacteriales order were identified. *Propionibacterium acnes* is first described as Hg resistant as well as its presence in rhizospheric soils.

Kribbella sp. is linked to both types of soil. Its resistance to Hg is not included in the scientific literature, although some authors have described strains of this genus resistant to other heavy metal divalent cations like Cu, Ni, and Cd (Chanda et al., 2017; Rosenfeld et al., 2018). Other authors describe the capacity of produce siderophores by some species (Acquah et al., 2020). The presence of this bacteria in a Hg-contaminated soil and the data found on the bibliography suggests postulating that species as a good target to look for strains with possible biotechnological potential for use in soil bioremediation.

Within the Geodermatophilales order, the Geodermatophilaceae family present in both types of soils was identified (**Table 1**). Although bacteria associated with the rhizosphere of some plant species have been described in the literature (Montero-Calasanz et al., 2017), it was found with a greater abundance in BS in the present study. There are references to some species in this family as resistant to heavy metals (Kou et al., 2018), although no evidence of their description as resistant to Hg has been found.

In the order of Micrococcales, two families and two species were detected (**Table 1**), *Agromyces* sp. and *Cellulomonas* sp.

TABLE 1 | General table of bacterial taxonomic identification, together with their relative abundances in BS and RS.

CLASS	ORDER	FAMILY	SPECIES	% RS	% BS	Ref Hg	Ref RS	PGPR	*N*	β-Lac
Acidobacteria	Acidobacterial	Acidobacteriaceae	Species	0	5.29	Vishnivetskaya et al., 2011	Kielak et al., 2016; Xu et al., 2018	–	–	Gonçalves and Santana, 2021
Actinobacteria	Solirubrobacterales	Family	Species	14.13	32.8	Gołębiewski et al., 2014	Hernández et al., 2015	–	–	Jauregi et al., 2021
	Propionibacteriales	Nocardioidaceae	*Kribbella* sp.	11.09	7.11	–	Álvarez-Pérez et al., 2017	Shan et al., 2018	Shan et al., 2018; Borah and Thakur, 2020	–
		Propionibacteriaceae	*Propionibacterium acnes*	0	5.42	–	–	–	–	Ramage et al., 2003
			Species	0	6.53	–	Yeager et al., 2017; Armanhi et al., 2018	–	–	–
	Geodermatophilales	Geodermatophilaceae	Species	3.56	7.79	–	Montero-Calasanz et al., 2017	Karray et al., 2020	–	–
	Micrococcales	Microbacteriaceae	*Agromyces* sp.	1.01	0	–	Muehe et al., 2015	Lee and Whang, 2020	Hu et al., 2021	Lee et al., 2021
		Cellulomonadaceae	*Cellulomonas* sp.	0.8	0	–	Zhao et al., 2018	Zhao et al., 2021	Suleiman et al., 2019	Zhang et al., 2021
	Micromonosporales	Micromonosporaceae	*Actinoplanes* sp.	0	8.62	–	Gkarmiri et al., 2017	Yamamoto et al., 2018; Kaur et al., 2021	Yamamoto et al., 2018; Kaur et al., 2021	Torres-Bacete et al., 2007
	Actinomycetales	Streptomycetaceae	*Streptomyces chartreusis*	0	1.13	–	Wang et al., 2019	Senges et al., 2018; Vurukonda et al., 2018; Wang et al., 2019	Vurukonda et al., 2018; Wang et al., 2019	–
	Streptosporangials	Thermomonosporaceae	Species	0	8.7	–	Malisorn et al., 2018	–	–	–
Cyanobacteria	Oscillatory	Microcoleaceae	*Microcoleus* sp.	0	5.54	–	Couradeau et al., 2019	Jan et al., 2018	Couradeau et al., 2019	Philippon et al., 2016
Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	*Mesorhizobium* sp.	4.98	0	Petrus et al., 2015	Garrido-Oter et al., 2018	Menéndez et al., 2020; Alemneh et al., 2021	Garrido-Oter et al., 2018; Menéndez et al., 2020	Rangel et al., 2017
			Mesorhizobium loti	6.79	0	–	Garrido-Oter et al., 2018	–	Garrido-Oter et al., 2018	–
		Bradyrhizobiaceae	*Afipia* sp.	3.46	1.94	Petrus et al., 2015	Jaiswal et al., 2017; Zheng et al., 2021	Jaiswal et al., 2017	–	–
			Rhodopseudomonas sp.	0	4.7	Deng and Jia, 2011	Wong et al., 2014; Hsu et al., 2021	Wong et al., 2014	Chang et al., 2020	–
			Rhodopseudomonas palustris	6.05	0	Deng and Jia, 2011	Wong et al., 2014; Hsu et al., 2021	Wong et al., 2014	Chang et al., 2020	McCully et al., 2018
		Hyphomicrobiaceae	Species	8.88	0	–	Xu et al., 2014	He et al., 2020	Martineau et al., 2014	Zheng et al., 2016

(Continued)

TABLE 1 | (Continued)

CLASS	ORDER	FAMILY	SPECIES	% RS	% BS	Ref Hg	Ref RS	PGPR	*N*	β-Lac
	Caulobacterales	Caulobacteraceae	*Caulobacter* sp.	4.5	2.24	Hu et al., 2005	Gutiérrez-García et al., 2019	Yang et al., 2019	Gutiérrez-García et al., 2019	Escandón-Vargas et al., 2017; Valencia et al., 2020
			Caulobacter vibrioides	0.67	0	Wang et al., 2016	–	–	Fatema et al., 2019	Escandón-Vargas et al., 2017
Betaproteobacteria	Burkholderiales	Comamonadaceae	*Variovorax* sp.	5.66	0	–	Belimov et al., 2015; Teijeiro et al., 2020	Toukabri et al., 2021	Woo et al., 2017; Toukabri et al., 2021	Dewi et al., 2020
Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas* sp.	7.9	0	Von Canstein et al., 1999	Zhuang et al., 2021	Zhuang et al., 2021	Geries and Elsadany, 2021	Falodun and Musa, 2020

Bibliographic reference of the Hg resistance of the taxon (Ref Hg); bibliographic reference of the presence in rhizosphere of the taxon (Ref RS); bibliographic activity reference as PGPR (plant growth promoting rhizobacteria) of the taxon; bibliographic reference of N-binding capacity, and bibliographic reference of the possession of β-lactam resistance genes (β-Lac).

Neither of these two species is cited as resistant to Hg, a fact that is first described in this study. Its resistance to other heavy metals is described (Corretto et al., 2017; Brookshier et al., 2018). The presence of *Agromyces* sp. associated with *Arabidopsis halleri* roots in the extraction of heavy metals by phytoextraction is not known (Muehe et al., 2015); the role of *Cellulomonas* sp. in plant rhizome as a producer of IAA is known (Zhao et al., 2018). The findings on these two bacteria are postulated by organisms with a possible biotechnological potential to look for strains for use in soil bioremediation in this study, despite their low representation in the data obtained (**Table 1**).

In the Micromonosporal order, a single species *Actinoplanes* sp. was detected, only in BS, being described as good PGPR (**Table 1**) (Gkarmiri et al., 2017; Yamamoto et al., 2018). Works such as that of Wang et al. (2019) have demonstrated the tolerance to various heavy metals by *Actinoplanes* sp.; no resistance references to Hg were found. The resistance to Hg of these species it's first described in this study.

The genus *Streptomyces* (Actinomicetales) is one of the most studied due to its high biotechnological and industrial potential, since a large quantity of antibiotics currently used clinically are derived from these bacteria (Hopwood, 2007). Its resistance to Hg has been referenced for more than 20 years (Ravel et al., 1998). *Streptomyces chartreusis* has been described as good PGPR (**Table 1**) (Senges et al., 2018; Vurukonda et al., 2018; Wang et al., 2019). This is the first time *S. chartreusis* has been referred to in a soil contaminated with Hg. The potential PGPR and its remediation capability noted in the literature, along with its presence in the study soil, suggests *S. chartreusis* as a good target for search strains that could be used in the remediation of this environment.

Cyanobacteria

Cyanobacteria (bacteria domain) include bacteria capable of performing oxygenic photosynthesis. It's a phylum that have an extensive ecological distribution, as well it's needed to take in account that the plots sampled are located in rainwater leaching areas and close to water sources, so it understandable to find this phylum on the sample (Mehetre et al., 2022). The species *Microcoleus* sp. was detected (**Table 1**). *Microcoleus* sp. has not been described as resistant to Hg. Godoy-Lozano et al. (2018) shows the high potential of this species to degrade various heavy metals. The data collected in the literature and their presence in these soils are indicative of their potential as research targets for its possible use in the bioremediation of Hg.

Alphaproteobacteria

Mesorhizobium sp. and *Mesorhizobium loti* (Phyllobacteriaceae) have been studied for their ability to form nodules in plant roots and fix N (Garrido-Oter et al., 2018). They are of great interest for their close relationship with the plant root and have been described as resistant to various heavy metals, such as Cd or Pb (Fan et al., 2018), highlighting their strong potential for use in soil remediation.

Afipia sp., *Rhodopseudomonas* sp. and *Rhodopseudomonas palustris* (Bradyrhizobiaceae): *Afipia* sp. arouses interest

for its potential use in remediation of soils contaminated with Hg (Petrus et al., 2015). *Rhodopseudomonas* sp. and *Rhodopseudomonas palustris* are described as PGPR (Wong et al., 2014; Hsu et al., 2021). Some authors (Batool and Rehman, 2017; Xiao et al., 2021) relate the PGPR capacity of *Rhodopseudomonas* sp. and *R. palustris* to their ability to remediate Hg-contaminated soils.

Calubacter sp. and *Caulobacter vibrioides* (Caulobacterales) have been described as resistant to various heavy metals because they host divalent cation ATPase transporters on their membranes (Hu et al., 2005). As for *C. vibrioides*, it has solubilizing activity of selenium used in processes of detoxification of Hg (Wang et al., 2016). As well, this bacterias appear in the bibliography described as nitrogen fixers (Gutiérrez-García et al., 2019). For these reasons, these bacteria have a biotechnological interest for use in future bioremediation pathways.

Betaproteobacteria

For this Class, only one specie could be identified. *Variovorax* sp. (Burkholderiales) has been characterized by Belimov et al. (2015), noted for its activity as PGPR and its resistance to various heavy metals, although not appearing in the literature as resistant to Hg. Having found this species in RS with high concentration of Hg postulates it as a good candidate to further studies to find strains from this species with potential use in bioremediation.

Gammaproteobacteria

Gammaproteobacteria are a diverse class of Gram-negative bacteria with a wide biological distribution. *Pseudomonas* sp. have a high biotechnological interest and have been studied for their characteristics as PGPR (Yasmeen et al., 2021). The bioremediation capacity of Hg is well known (González et al., 2021; Imron et al., 2021; Robas et al., 2021a). Therefore, a study of its potential use in the remediation of contaminated soils and look for non-phytopathogenic specific strains is of interest.

Viruses

Two viral families were identified in the metagenome, Circoviridae and Potyviridae. The first is a family that is distributed in the environment infecting mainly vertebrates (Delwart and Li, 2012). Only the species Cyclovirus NGChicken15/NGA/2009was identified, it is a virus typically pathogenic from farm birds. It is interesting and remarkable that of the 21% of the relative taxonomic abundance of viruses in the RS sample, 18.88% belong to a single virus that is very far from its natural host. Due to the ubiquity of this virus and its presence in our sample only in RS we suspect that it could be a virus very similar to NGchicken15 that is infecting the plant or any of the bacteria in the rhizosphere such as prophage.

From the family Potyviridae was identified the species Dasheen mosaic virus, very common species pathogenic of vegetables (Francki et al., 2012) with a similar relative abundance in both soils (1.66% RS and 2.19% BS) It is not strange to find this species both in the fraction of SL and RF given that the samples were collected at a time of high growth and therefore of a greater transmissibility of this virus to plants and to the soils.

Functional Analysis

Functional potential bioinformatic analysis allows the identification of potential metabolic activities and processes. In this way, it is possible to sort the potential functions of the microbial community according to their abundance. However, minority activities should be taken into account as long as they allow the biological behavior of these communities to be interpreted. In this sense, most of the functional potential clusters identified, and sequences associated to a protein belong to basal metabolism. However, the mechanisms of Hg resistance, those involved in oxidative stress, N metabolism and PGPR activity are more important from an ecological and functional potential point of view. In addition, sequences associated to a protein have been found whose presence in soils can only be interpreted as indicating biological contamination (Li et al., 2020; Stange and Tiehm, 2020). Since there is no antibiotic pressure on the analyzed soils, the mechanisms of resistance commonly described in clinical studies should not be detected in environmental samples. For this reason, in the area of "One Health" they deserve to be analyzed and interpreted as bioindicators of biological pollution. Microbial soil communities can act as reservoirs from which information can be transferred horizontally to potential pathogens, becoming a threat to human, animal and environmental health (Wang et al., 2021).

Antibiotic Resistance

Soils, especially those under high environmental pressure, act as a natural reservoir of resistance to existing antibiotics or provide the potential to host bacteria of clinical importance (Yan et al., 2020; Liao et al., 2021). Several studies show the existence of a co-choice between the presence of various toxic compounds in the environment and the selection of antibiotic resistance naturally, together with co-resistance to heavy metals and antibiotics (Yan et al., 2020; Mazhar et al., 2021; Robas et al., 2021b).

At level II this functional cluster were more represented in RS than in BS. At level III of the metagenome study, several specific role clusters for resistance to rare antibiotics in the natural environment have been identified (**Supplementary Table 12**), such as β-lactamases and mechanisms associated with resistance to beta-lactams and predicted proteins associated with gene responses to these antibiotics BlaR1 and MecR1 (Silveira et al., 2018).

Likewise, the presence of various transporters dependent on ATP of Pb, Cb, Cu, and Hg in the group of functional activities regulating the beta-lactams BlaR1 was found, and a direct relationship between the presence of these heavy metals and β-lactam resistance. Among the predicted proteins related with β-lactam resistance genes isolated in this study, some belonging to classes A, C, and D were identified.

Class A includes several subsystems, all fundamentally linked to ampicillin resistance, that are rarely used clinically owing to numerous described resistances that exist for this antibiotic (Kaye et al., 2000; Rice et al., 2001). CTX-M-16 was found, which gives greater catalytic power than other cefotaximes (Bonnet et al., 2001). The finding in the present work of resistance mechanisms to these antibiotics was evidence of the selection of resistance

mechanisms of clinical origin, especially when occurring in semi-synthetic compounds that do not occur in nature.

The AmpC-type β-lactamases are of great clinical importance because they are hydrolyzed penicillins, cefamycins, oxyminocephalosporins and monobactams, although they are not active against fourth-generation carbapenemic cephalosporins (Jacoby, 2009; Aguirre et al., 2020). The relevance of these genes is provided by their high transmissibility, since many are found in plasmids (Ku et al., 2019; Rensing et al., 2019).

The MexAB-OprM and MexEF-OprN complexes are a protein assembly of membrane transporters that provide multi-resistance to antibiotics, identified primarily in *Pseudomonas* sp. and are highly linked to multi-resistance in *P. aeruginosa* (Ma et al., 2021). As shown in **Table 1**, there is high probability that these genes that could confer multi-resistance can be associated with the *Pseudomonas* identified in this manuscript. In the analysis of the sequences, were predicted proteins from the MexT regulator was found, which is inactivated by some Hg resistance genes, making the strains carrying the MexEF-OprN complex sensitive to carbapenems (Köhler et al., 1999).

At level III, sequences related to RND transporters have been found in multi-resistance efflux pumps functional potential cluster. Among these, there is a large representation of MexAB-OprM and MexEF-OprN, along with MexCD-OprJ antibiotic transporters, related not only to antibiotic resistance but also mediate processes of quorum sensing (Alcalde-Rico et al., 2018). Other antibiotic resistance predicted proteins found were those from AcrAB-TolC, a system responsible for the expulsion of antibiotics, such as penicillin G, cloxacillin, naphthyllin, macrolides, novobiocin, linezolid, and fusidic acid derivatives; this system is commonly associated with E. coli (Anes et al., 2015; Byrd et al., 2021). Proteins from MdtABC genes have also been predicted in SEED, related TolC, which give resistance to novobiocin, quinolones and phosphomycin, among others (Anes et al., 2015). CmeABC membrane transporters were also found, related to Campylobacter jejuni's resistance to a wide variety of antibiotics (Lin et al., 2002; Yan et al., 2006), although authors such as Stopnisek et al. (2016) have reported the presence of these genes in other species within the Rhizobiales order.

Some proteins directly related to antibiotic multi-resistance mechanisms have been predicted with the metagenome sequences in Gram-positive bacteria cluster. The MdtRP operon of *Bacillus* sp. confers resistance to several antibiotics such as novobiocin, streptomycin and actinomycin, and is regulated by the MarR repressor (Gupta et al., 2019; Warmbold et al., 2020).

Another of the functional subsystems identified in level III and SEED is related to tripartite protein expulsion systems in Gram-negative bacteria. Similar to the already noted MexAB-OprM or AcrAB-TolC, they belong to membrane transporters RND (Daury et al., 2016). No specific identification of any of these sequences was achieved beyond identifying them as antibiotic ejection systems within the RND group and related to tripartite proteins such as MdtABC, and ejection proteins from genes such as TolC or OprN. This highlights a variety of resistance systems that bacteria can possess in a hostile environment, and which are still unknown.

An important functional cluster related to fluoroquinolone resistance has been found. These are synthesis compounds, not found naturally in environmental samples. Hooper (2000) describes how fluoroquinolone resistances are acquired by mutations in the genes of Topoisomerases II and IV, predicted proteins present in the metagenome sequences of the samples analyzed at level III and SEED. Similarly, pumps from Lde genes were found, which are specific to fluoroquinolones (Godreuil et al., 2003).

A functional cluster and predicted proteins related with vancomycin resistance were also found. The proteins predicted VanA, VanB, VanH, VanR, VanS, VanW, VanX and VanZ, all of which were directly related to vancomycin resistance (Qureshi et al., 2014; Stogios and Savchenko, 2020) and VanZ, which in turn gave teicoplanin resistance (Qureshi et al., 2014) and VanW whose role in resistance mechanisms is still unknown. These genes are usually grouped by their function and level of resistance, thus taking the VanAB, VanHAX, and VanRS groups (Bugg et al., 1991; Arthur et al., 1992). The presence of some sequences that predict proteins, such as those related with VanAB genes, appearing in plasmids has been studied (Ishihara et al., 2013; Qureshi et al., 2014; Sivertsen et al., 2016), giving this resistance greater potential to be transmitted to other species. Several proteins related with that regulation of the activity of this resistance were also found, such as VanRS and a histidin-kinase system that activates the resistance system (Arthur and Quintiliani, 2001; Qureshi et al., 2014; Stogios and Savchenko, 2020).

Another group of resistance functional potential cluster was that corresponding to bacitracin resistance. The predicted proteins in SEED correspond to ABC flow pumps associated with the *Bacillus* genus such as BceAB, BceR, YvcPR, YxdM, YclH, YknY, BseL and LiaRS (Mascher et al., 2004; Kingston et al., 2014). Some of these genes have also been reported in other Gram-positives, such as some species of *Enterococcus* and *Clostridium* (Charlebois et al., 2012; Zhou et al., 2019).

Hg Resistance and Oxidative Stress Response

The mechanisms of resistance to Hg are widely described in the microbial world. For this reason, it is not uncommon to detect various predicted proteins associated with genes from operon *mer* (MerC, MerE, and MerT) (Gionfriddo et al., 2020) and mercuroreductases in the samples analyzed (**Supplementary Table 6**). In the same way, some resistance and transport mechanisms for divalent cations (Co, Zn, and Cd primarily) (**Supplementary Table 6**) were found, revealing the probably participation of that mechanisms in the resistance to Hg of the microorganisms. Likewise, ABC-type transporters (ATP-binding cassette), capable of providing resistance to bacteria against various toxins (Acar et al., 2020; Thomas and Tampé, 2020), account for almost 50% of membrane transporters in level II. At SEED level it can be found some ABC and RND efflux systems related with resistance with divalent cations and heavy metals (**Supplementary Table 6**). These transporters are widely distributed throughout the metagenome and primarily associated with resistance systems, acting as efflux pumps for different toxic compounds (**Supplementary Table 3**).

Environmental factors are known to cause oxidative stress to the colonizing microorganisms of contaminated soils. In order to colonize these environments, bacteria need effective biological mechanisms. Stress response genes give microorganism methods of adaptability to host situations and environments that may affect their normal development (Chakraborty and Kenney, 2018; Dweba et al., 2018; Kokou et al., 2018). These potential functional activities are found with a high relative abundance (**Supplementary Table 7**), as the soil is a "nutritional desert" and has the abiotic stress of high Hg concentration. These factors are in a similar proportion in both samples; it seems that BS and RS exert a different environmental pressure on organisms in terms of stress. Various ABC transport mechanisms have been developed, which function as part of the stress response machine in a hostile environment (Teichmann et al., 2018; Van Goethem et al., 2018). As well some sequences related with the carbon starvation, like proteins of Slp (Alexander and St John, 1994) and Sspa (Yin et al., 2021) were found. And functional potential clusters related with weather conditions (Cold and heat shock, desiccation stress and osmotic stress) (**Supplementary Table 7**).

N Metabolism and PGPR

N is a limiting macronutrient for the proliferation of microorganisms and the growth of plants. Therefore, the enzymes involved in the synthesis of nitrogen compounds usable by plants are relevant. In the present study, in level I was found a functional potential cluster that involves the N metabolism (**Supplementary Table 8**). Several sequences have been associated with proteins related to the assimilation of nitrate and nitrite as the Nar (NarA, NarD, NarE, NarK, NarL, and NarP) (Fukuda et al., 2015). Among the N metabolism, proteins from Nir denitrification genes were identified. Some of these sequences, related with genes such as NirT and Nos, are involved in N monoxide denitrification and formation (Bergaust et al., 2012; Belbahri et al., 2017). At level III, was alsa found a cluster related with the nitrogen fixation, fundamentally from the operon Nif were identified, which codes for the nitrogenase complex, fundamental in the fixation of atmospheric N in the soil (Di Cesare et al., 2018; Dasgupta et al., 2021; **Supplementary Table 8**).

Plant growth-promoting bacteria are characterized by their ability to produce and/or adapt to a hostile environment, such as auxin production, phosphate solubilization, ACC degradation, and siderophore production. Several sequences clustered in potential functional activities involved in the synthesis of auxins, an important factor promoting the growth of plants, have been identified (Mishra et al., 2021). Specifically, 3-indolacetic acid (IAA) is related to a large number of processes that improve plant quality. Some proteins from the principal biosynthetic routes of IAA, indole-3-pyruvate and indole-3-acetamide were predicted (Nascimento et al., 2021), such as proteins IorA and IorB (**Supplementary Table 9**). Likewise, IAA has the capacity to improve the tolerance of plants to adverse conditions and stress by heavy metals (Ma et al., 2011; Nazli et al., 2021). Some authors (Ma et al., 2011; Zainab et al., 2020) have established a relationship between rhizospheric bacteria producing IAA and significant improvement together with greater speed in phytoextraction of heavy substances and recovery of contaminated soils.

Plants and microorganisms compete for phosphorus present in the environment; therefore, the solubilization of phosphorus by microorganisms contributes to the promotion of plant growth (Pereira et al., 2020). A large number of proteins genes have been predicted for acid phosphatases, phytases, gluconate dehydrogenases, ketogluconate dehydrogenases, and glucose-1-dehydrogenase (**Supplementary Table 10**), which are encompassed in the context of phosphate solubilization (Suleman et al., 2018).

The predicted proteins of the AcdS genes of the ACC deaminase (1-aminocyclopropane-1-carboxylate desaminase) found in our metagenome interfere with the synthesis of ethylene in the plant by degradation of a metabolic precursor, thereby reducing stress in the tissues (Glick, 2014; del Carmen Orozco-Mosqueda et al., 2020). Ethylene is a marker of plant stress and senescence, so this enzyme helps plants withstand stressful environments, such as soils contaminated with heavy metals.

A wide variety of siderophore functional potential clusters were also identified (**Supplementary Table 11**), it can be found at level II a functional potential cluster related with siderophores (**Supplementary Table 3**). Siderophores act as metal chelators favoring, among other potential functions, the absorption of iron and its entry into the food chain. Plants that are able to use bacterial siderophores as a source of iron (Wang et al., 1993) increase their chances of survival and adaptation to contaminated environments.

CONCLUSION

Several conclusions can be drawn from this study.

In the taxonomic analyze, the most abundant microbial genome analyzed belongs to the bacteria domain. The prevalent taxa are those of Actinobateria and Alphaproteobacteria. Betaproteobacteria and Gammaproteobacteria seems to be intimately linked to rhizospheric soil (RS). Likewise, Cyanobacteria and Acidobacteria only have representation in bulk soil (BS). Similarly, the genome belonging to the Domain Eukarya involved in the potential functional activity of microbial activity was detected. The viral genomes present in the sample are interpreted as prophages.

On the functional potential profiling, the presence of antibiotic resistance mechanisms and other toxic compounds could confirm previous studies pointing to their co-selection in Hg-contaminated environments. The finding of resistance mechanisms proper to human clinical, evidence of biological contamination, suggests that these environments may behave as reservoirs. Although, the presence of Hg resistance functional clusters and involved in the response to oxidative stress are present as a minority; however, their biological significance justifies the behavior of the microbial community. The abundance of PGP and N-fixation functional activities detected in the metagenome sequences may be an opportunity for further selection of both effective bioremediation strains and genes for promoting biotechnological use in the production of GMOs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AP and PJ supervised the project and acquired funding for this research. All authors designed the experiments, made intellectual contributions, conducted the experiments, analyzed the data, wrote and edited the manuscript.

FUNDING

This research has been funded by Fundación Universitaria San Pablo CEU (Microbiology SAI) and Banco Santander, grant number FUSP-BS-PPC01/2014.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.797444/full#supplementary-material

Supplementary Table 1 | Relative abundances at all taxonomical levels (Metaphlan data table).

Supplementary Table 2 | Relative abundances of SUPERFOCUS functional level I.

Supplementary Table 3 | Relative abundances of SUPERFOCUS functional level II.

Supplementary Table 4 | Relative abundances of SUPERFOCUS functional level III.

Supplementary Table 5 | Relative abundances of SUPERFOCUS at SEED function level.

Supplementary Table 6 | Relative abundances of SUPERFOCUS functional levels I–III and SEED of resistance to heavy metals potential functional activities.

Supplementary Table 7 | Relative abundances of SUPERFOCUS functional levels I–III and SEED of stress response and oxidative stress potential functional activities.

Supplementary Table 8 | Relative abundances of SUPERFOCUS functional levels I–III and SEED of nitrogen metabolism potential functional activities.

Supplementary Table 9 | Relative abundances of SUPERFOCUS functional levels I–III and SEED of IAA potential functional activities.

Supplementary Table 10 | Relative abundances of SUPERFOCUS functional levels I–III and SEED of ACCd potential functional activities.

Supplementary Table 11 | Relative abundances of SUPERFOCUS functional levels I–III and SEED of siderophores potential functional activities.

Supplementary Table 12 | Relative abundances of SUPERFOCUS functional levels I–III and SEED of resistance to antibiotics potential functional activities.

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2. Selección de cepas mercurotolerantes con capacidad promotora del crecimiento vegetal

El uso de bacterias promotoras del crecimiento vegetal (PGPB; *Plant Growth Promoting Bacteria*) persigue la mejora y/o aceleración del crecimiento de las plantas debido a la capacidad de las bacterias para solubilizar fósforo, fijar nitrógeno y sintetizar sideróforos, además de producir fitohormonas (auxinas, giberelinas, citoquininas) o sustancias moduladoras del estrés.

En este trabajo se planteó un *screening* de cepas bacterianas. Sus actividades PGPB se midieron en medios con diferentes concentraciones de Hg, pudiendo establecer como concentración de Hg óptima para la mediad de las actividades, y posterior selección de las cepas bacterianas, 100 µg/mL. Así mismo se obtuvo su CMB frente a dicho metal. Los datos obtenidos de cada cepa se ponderaron usando el Índice de Idoneidad Biomercuroremediador (IIBRM). Usando dicho índice se seleccionaron cuatro cepas con un alto potencial que se emplearían en los sucesivos ensayos: *Bacillus toyonensis*, *Pseudomonas moraviensis* (posteriormente renombrada como *Pseudomonas mercuritolerans sp nov*), *Pseudomonas baetica*, y *Brevibacterium frigoritolerans*. Todas estas cepas poseían un IIBRM superior a 6,5 (puntuación establecida por el grupo de investigación para que la cepa sea considerada de alto potencial PGPB), presentaban una CMB > 140 µg/mL y tenían una capacidad de producción de auxinas mayor a 6 µg/mL.

En esta publicación, realicé el todo el trabajo experimental y posterior análisis de datos. Del mismo modo, escribí el primer borrador del manuscrito, sobre el que mis directores de tesis fueron aportando sugerencias que incorporé en sucesivas revisiones previo al envío a la revista. Fui el encargado del proceso de envío y comunicación con la revista durante todo el proceso de revisión y publicación.

Factor de impacto (2020): 3.390. **Categoría:** Public, Enviromental and Occupational Health (Q1 45/182). **Fuente**: JCR (Journal of citation reports)

International Journal of
Environmental Research and Public Health

MDPI

Article

Selection of Mercury-Resistant PGPR Strains Using the BMRSI for Bioremediation Purposes

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Abstract: Heavy metal pollution of soil, particularly by mercury (Hg), is a problem that can seriously affect the environment and human health. For this reason, it is necessary to take steps to remediate these environments, prevent potential adverse effects, and restore these areas for subsequent use in agriculture, industry, ranching, and forestry. The present study has selected 40 bacterial strains from rhizosphere and bulk soil that grow naturally in high Hg-contaminated soils from the Almadén mining district in Ciudad Real, Spain. With the objective of evaluating the potential use of these strains in phyto-rhizoremediation, an evaluation and statistical analysis of their PGPR (Plant-Growth-Promoting Rhizobacteria) activity at different levels of Hg was carried out as the first condition of selection for their potential use in bioremediation. In addition, a Hg MBC (Maximum Bactericidal Concentration) was performed with the aim of selecting the strains with high Hg tolerance. Finally, strains with potential biotechnological use have been proposed according to the Bio-Mercury Remediation Suitability Index (BMRSI) criteria, which consider indole-3-acetic acid (IAA) production, acid 1- aminocyclopropane-1-carboxylic deaminase (ACCd) activity, phosphates solubilization, and siderophore production measured in the presence of Hg, as well as its MBC to Hg. The strains selected for further in vivo and in situ processes must reach at least an MBC (Hg) > 100 μg/mL and BMRSI ≥ 6.5.

Keywords: heavy metal pollution; bioremediation; PGPR; BMRSI; mercury

Citation: González, D.; Robas, M.; Probanza, A.; Jiménez, P.A. Selection of Mercury-Resistant PGPR Strains Using the BMRSI for Bioremediation Purposes. *Int. J. Environ. Res. Public Health* **2021**, *18*, 9867. https://doi.org/10.3390/ijerph18189867

Academic Editor: Paul B. Tchounwou

Received: 3 August 2021
Accepted: 15 September 2021
Published: 18 September 2021

1. Introduction

Mercury (Hg) is an element with a high level of toxicity that poses a serious environmental threat. It is possible for Hg to enter the food chain and consequently affect human health, even at very low concentrations [1,2]. From a toxicological point of view, Hg is a toxic metal without any specific biological function. This element has a high potential for bioaccumulation and biomagnification due to the high solubility of Hg and methylmercury in fat and muscle tissue. An accumulation of Hg can result in pathologies of the central nervous system, such as Minamata's Syndrome [3], as well as other health problems related to development, growth, and fertility [4].

On a global scale, anthropogenic emissions add approximately 2500 Mg of Hg to the atmosphere every year. In Europe, Lado et al. [5] developed a model of Hg distribution in the soil in 28 countries. According to their research, the average amount of Hg in European soil was around 40 μg kg^{-1}. Soils in Northern Europe have a higher concentration of Hg than soils in the countries of Central and Southern Europe due to the fact that cold, wet weather promotes the accumulation of Hg in organic matter in soil [6]. Studies related to Hg distribution in soils adjacent to the Hg mines in the Almadén mining district reveal the presence of both high and extremely high levels of Hg (up to 8889 μg/g),

while the concentration in sediment and water reaches levels of up to 16,000 μg/g and 11.2 μg/L, respectively [7].

In 2003, after more than 2000 years of activity, the mines closed due to a decrease in the demand for Hg, as well as changes in European regulations regarding this metal. With the aim of providing alternative uses for the Almadén soil, the scientific community has been working to develop strategies to mitigate the effects of Hg. Certain physicochemical methods have been developed that enable the elimination of this metal from soil, but the current trend is to use biological methods that are more environmentally friendly, based on biotechnological techniques such as bioremediation. This is the case with phyto-rhizoremediation, which involves the synergistic collaboration of plants and microorganisms for the purpose of remediating chemical compounds and pollutants from the environment [8]. An example of this activity is the use of plant growth-promoting rhizobacteria (PGPR) [9], which can be used in phyto-rhizoremediation aimed at the plant's root in order to aid its physiological development, as well as direct activity aimed at the pollutant, while simultaneously increasing the effect of the plant itself on the pollutant.

Hg tolerance and Hg resistance of microorganisms can contribute to the reduction and/or elimination of the different types of Hg in contaminated environments, which has led to increased interest in the selection of bacterial strains with biotechnological potential, as well as their use in bioremediation [10].

The Bio-Mercury Remediation Suitability Index (BMRSI) has proven to be a useful tool for evaluating the Hg bioremediation potential of the bacterial strains, since it takes into account not only the Hg resistance capability of the bacteria, but also their combined PGPR capacity. For this reason, the present study proposes a BMRSI analysis of the best forty bacterial strains obtained by Robas et al. [11] in the presence of Hg.

2. Materials and Methods

2.1. Bacteria Analyzed

This study was carried out with samples from the Almadén mining district in Ciudad Real, Spain. An experimental plot was used (*Plot M)*, as it has been classified as an area of high Hg contamination with concentrations of 1710 mg/kg Hg [12]. The plants used for the bacterial extraction from the rhizosphere were the following: *Rumex induratus* Boiss. and Reut., *Rumex bucephalophorus* L., *Avena sativa* L., *Medicago sativa* L., and *Vicia benghalensis* L., in addition to bulk soil. All the plant species were sampled in situ at *Plot M* during the spring season, looking for the maximum biological activity in that region. The plant samples were carried to the laboratory at 4 °C and processed before 24 h.

The bacteria selected for the study were isolated, characterized, identified, and selected as effective Hg remediators by measuring their capabilities as PGPR in the absence of Hg, by Robas et al. [11], as shown in Table 1. All the isolated strains were stored at −80 °C before their use in the present study.

Table 1. Strains analyzed with their corresponding BMRSI values, obtained by Robas et al. [11].

Strain	1	9	10	11	18	20	21	23	31	35	37	43	48	50
BMRSI	6.68	6.56	7.42	7.69	7.88	7.56	7.21	6.97	7.40	5.02	7.07	7.69	6.62	7.09
Strain	55	56	57	58	69-I	69-II	70	74	75	76	79	80	95	98
BMRSI	7.23	6.43	7.26	6.46	7.86	8.51	6.35	8.07	6.30	7.04	7.55	8.42	7.57	7.05
Strain	114	122	130	146	149	160	173	175	204	211	211-I	214	114	122
BMRSI	7.67	6.59	8.01	7.99	6.26	6.32	6.60	7.08	6.80	7.74	7.64	5.40	7.67	6.59

2.2. Testing PGPR Activity

Each PGPR activity was tested according to the protocols described in the bibliography. These protocols were modified in an innovative way in order to test the

PGPR capacity of the isolates in the presence of different concentrations of Hg. The objective was to validate this method of analyzing PGPR activity[11] in the presence of heavy metal by using the BMRSI.

The concentrations of Hg tested in each protocol were 80 μg/mL, 100 μg/mL, 120 μg/mL, and 140 μg/mL of Hg Cl_2.

To determine the production capacity of Indole-3-Acetic Acid (IAA) in vitro, a colorimetric technique with the reagent Van Urk Salkowski from the Salkowski method was used [13]. The isolated bacteria were grown in LB broth (Lennox) with the proposed protocol modification and incubated at 28 °C for 48 h with the IAA concentration measured at intervals of 12 h, 24 h, and 48 h. The results were quantified in μg/mL.

To determine the ability of the strains to degrade acid 1- aminocyclopropane-1-carboxylic (ACC) through the activity of ACC deaminase, the protocol described by Glick [14] was followed and modified, as described above.

The siderophore production was determined by the use of Chrome Azurol S (CAS) agar described by Alexander and Zuberer [15] and modified by the addition of Hg.

The ability to solubilize inorganic phosphates was determined by the use of the protocol described by de Freitas et al. [16] and modified, as in the previous case.

2.3. Maximum Bactericidal Concentration of Hg (MBC)

To study the Hg MBC, the selected bacteria were seeded on Müller Hinton agar plates of the commercial brand Pronadisa® (Eucast, 2017, Växjö, Sweden) following the protocol and criteria established by Robas et al. [11].

2.4. Bio-Mercury Remediation Suitability Index (BMRSI)

To evaluate the bio-mercury remediation potential of the strains, the BMRSI proposed by Robas et al. [11] was used. BMRSI measures the bioremediation potential of the strains by the inclusion of different PGPR activities and its Hg MBCs in one formula:

$$BMRSI = [IAA\ (\mu g/mL) + ACCd\ (1/0) + SID\ (cm) + PO_4^{3-}\ (1/0)] + [MBC\ Hg\ (\mu g/mL)]$$

where: Presence = 1; Absence = 0.

2.5. Data Processing

Using the results of the auxin production, descriptive statistical analyses were carried out using the SPSS v26.0 program (Version 26.0 IBM Corporation). The purpose of these analyses was to ensure that the modification of the protocols for this study provides statistical significance to the data obtained in the experiments. In order to determine which group of IAA production data based on the Hg concentrations tested would be subsequently included in the BMRSI, a statistical ANOVA analysis was performed at each of the intervals tested (12 h, 24 h, and 48 h). When a significance level of $p < 0.05$ was obtained, a post hoc analysis was then carried out using the Bonferroni test.

3. Results

The selected strains were subjected to more extensive tests in order to identify the best candidates for further uses in phyto-rhizoremediation based on their PGPR capabilities in the presence of Hg.

For this purpose, only the data obtained in the Hg tests were analyzed due to the fact that the final objective of this study was to analyze the remediation capability in the presence of Hg, as well as the choice of the best strains for use in the bioremediation of plots contaminated with this heavy metal.

Figure 1 shows the trend of IAA production at different Hg concentrations over time (12 h, 24 h, and 48 h) in all the strains. Data measured at 12 h and 24 h were found to be significantly higher than those measured at 48 h ($p < 0.05$). By analyzing the mean values, it was found that during 12 h incubation period, the production of IAA was significantly higher at concentrations of 80 μg/mL and 100 μg/mL than at 120 μg/mL and 140 μg/mL

of Hg ($p < 0.05$). However, in the incubation period of 24 h and 48 h, at concentrations of 80 µg/mL, 100 µg/mL, and 120 µg/mL, IAA production was significantly higher ($p < 0.05$ and $p < 0.005$, respectively), than at concentrations of 140 µg/mL Hg.

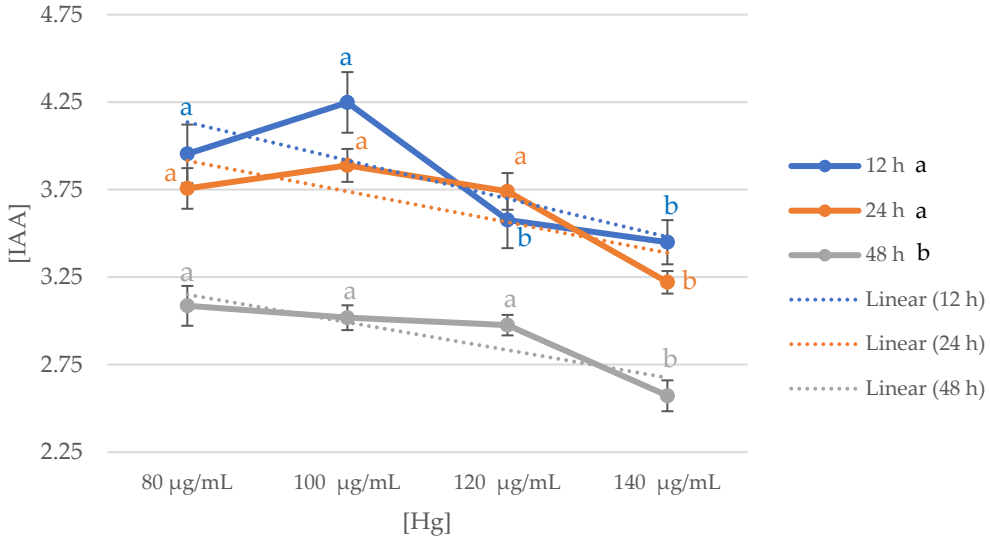

Figure 1. Average IAA production trend of the forty strains studied in relation to Hg concentrations at each of the measurement times. Letters a,b indicate the significance of $p < 0.05$, the black letters indicate significance differences among those grouped by hours; the blue letters indicate significance differences among the different concentrations of IAA measured at 12 h; orange letters indicate significance differences among different concentrations of IAA measured at 24 h; grey letters indicate significance differences among different concentrations of IAA measured at 48 h.

Therefore, it can be concluded that the average production value of the strains analyzed is obtained between 12 h and 24 h at concentrations between 80 µg/mL and 100 µg/mL. To select the range of data to be used later, values corresponding to the 12 h incubation period in mediums with a concentration of 100 µg/mL were used as a reference.

As shown in Table 2, only five strains (9, 48, 58, 122, and 173) exhibited ACCd activity. All of these showed activity up to concentrations of 100 µg/mL, and two of them (strains 9 and 58), up to 120 µg/mL of Hg.

Only three strains (50, 57, and 69-II) solubilize phosphates under the Hg conditions studied.

The production of siderophores was not included in Table 2 since no strain is safe to produce in the presence of Hg.

Regarding MBC, all strains resisted concentrations above 100 µg/mL. The minimum concentration resisted by the 40 strains was 140 µg/mL. A total of 55% of the strains tested resisted up to 140 µg/mL, but the other half had much higher resistance values. In the remaining 45%, we found nine strains that resisted up to 160 µg/mL, two up to 180 µg/mL, four up to 200 µg/mL, and three of them resisted up to 350 µg/mL.

Finally, after the analysis was carried out for each of the variables, the BMRSI was calculated using the data of the PGPR activity measured at 100 µg/mL in order to introduce the least possible variability and obtain uniform data from the sample.

Table 2 shows the integrated data of the PGPR and MBC activities of the 40 strains considered for evaluation using the BMRSI.

Table 2. Strains ordered by BMRSI descending values with all factors integrated, IAA: IAA production; PO_4^{3-}: solubilization of phosphates; ACCd: degradation of ACC via ACC deaminase; MBC: maximum bactericidal concentration. 0/1 indicates absence/presence. ND: not defined bacteria.

Strain	Identification	IAA (μg/mL)	PO_4^{3-}	ACCd	MBC (μg/mL)	BMRSI
9	*Bacillus toyonensis*	6.16	0	1	140	7.30
21	*Pseudomonas moraviensis*	7.06	0	0	140	7.20
98	*Pseudomonas baetica*	6.76	0	0	160	6.92
95	*Brevibacterium frigoritolerans*	6.40	0	0	140	6.54
37	*Pseudomonas fluorescens*	6.08	0	0	140	6.22
56	*Pseudomonas brassicacearum subsp. brassicacearum*	6.05	0	0	160	6.21
58	*Pseudomonas brassicacearum subsp. brassicacearum*	4.70	0	1	160	5.86
31	*Pseudomonas brassicacearum subsp. brassicacearum*	5.67	0	0	140	5.81
122	*Brevibacterium frigoritolerans*	4.37	0	1	160	5.53
50	*Bacillus toyonensis*	4.15	1	0	350	5.50
173	*Bacillus toyonensis*	3.93	0	1	180	5.11
48	*ND*	3.91	0	1	140	5.05
57	*Pseudomonas corrugata*	3.61	1	0	350	4.96
55	*Pseudomonas syringae pv. phaseolicola*	4.80	0	0	140	4.94
69-II	*Pseudomonas sp.*	3.77	1	0	160	4.93
70	*Pseudomonas corrugata*	4.51	0	0	350	4.86
69-I	*Pseudomonas syringae pv. phaseolicola*	4.67	0	0	160	4.83
43	*Bacillus toyonensis*	4.59	0	0	160	4.75
1	*Pseudomonas migulae*	4.59	0	0	140	4.73
23	*Pseudomonas moraviensis*	4.42	0	0	140	4.56
76	*ND*	4.10	0	0	140	4.24
204	*Brevibacterium frigoritolerans*	4.04	0	0	160	4.20
149	*Pseudomonas syringae pv. phaseolicola*	4.02	0	0	140	4.16
211	*Bacillus dendretensis*	3.85	0	0	200	4.05
114	*Pseudomonas syringae pv. phaseolicola*	3.80	0	0	140	3.94
75	*Pseudomonas syringae pv. phaseolicola*	3.74	0	0	160	3.90
79	*Pseudomonas syringae pv. phaseolicola*	3.66	0	0	140	3.80
74	*Xanthomonas oryzae pv. oryzae*	3.66	0	0	140	3.80
35	*Pseudomonas baetica*	3.64	0	0	140	3.78
20	*Pseudomonas fluorescens*	3.64	0	0	140	3.78
175	*ND*	3.50	0	0	140	3.64
130	*Pseudomonas corrugata*	3.47	0	0	140	3.61
18	*Bacillus toyonensis*	3.45	0	0	140	3.59
11	*Pseudomonas corrugata*	3.34	0	0	200	3.54
146	*Pseudomonas fluorescens*	3.20	0	0	180	3.38
10	*ND*	3.09	0	0	140	3.23

160	*Bacillus circulans*	3.09	0	0	140	3.23
211	*Bacillus dendretensis*	2.88	0	0	200	3.08
214	*Bacillus niacini*	2.82	0	0	200	3.02
80	*Pseudomonas syringae pv. phaseolicola*	2.85	0	0	140	2.99

When the measurement is standardized at 100 μg/mL of Hg, it can be observed that the datum with greater weight in the calculation is the amount of IAA produced by each strain. Similarly, the production of siderophores for all the strains in the selected conditions is 0. Therefore, strains with high IAA production that exhibit other PGPR activity will have a higher BMRSI. Similarly, the taxonomic identification of the 40 selected strains can be observed in Table 2 [11].

As such, a percentage comparison was made of the number of bacteria that exhibit each of the PGPR activities at 0 μg/mL of Hg obtained by Robas et al. [11], compared to those obtained using the selection criterion of 100 μg/mL of the present study, as shown in Figure 2. As can be seen in Figure 2, a reduction in the PGPR capacity of the bacteria under study occurs when these activities are analyzed in the presence of Hg.

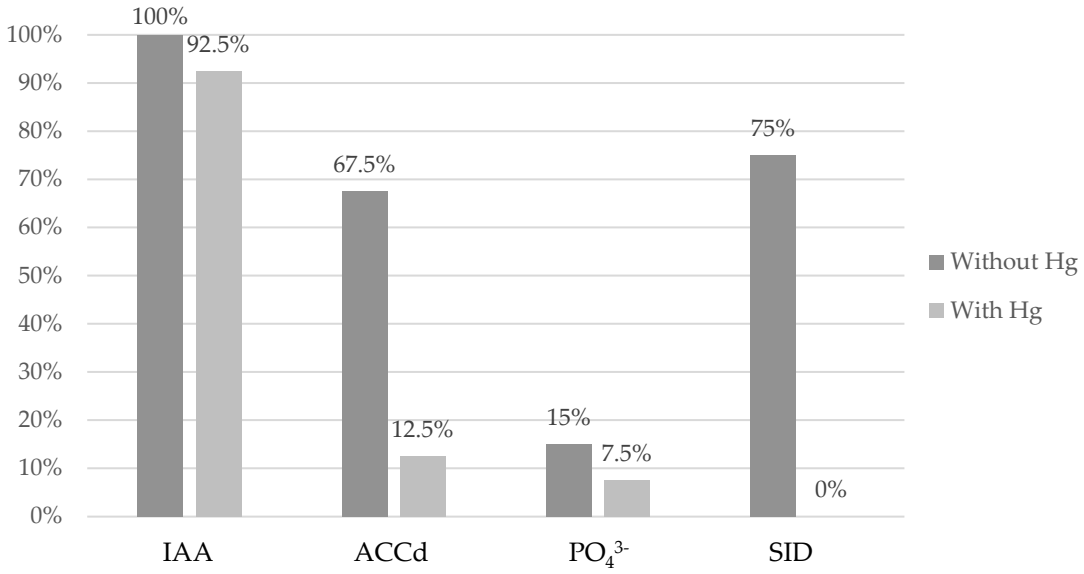

Figure 2. Percentage comparison of the data obtained by Robas et al. [11], contrasted with those obtained in the present study of the number of strains with PGPR activity at 0 μg/mL and 100 μg/mL of Hg. IAA: auxin producers; ACCd: ACC degraders; PO_4^{3-}: phosphate solubilizers; and SID: siderophore producers.

4. Discussion

The fraction of soil surrounding the plant roots (rhizosphere) provides an environment that enables the growth of a large number of microorganisms [17]. Among these microorganisms, PGPRs have been shown to assist plant growth [18]. In addition, those that were able to support plants in their phytoremediation activity against heavy metals, including Hg [19–23], are of particular interest for the present study. In this research, we have selected and classified PGPR strains based on their quantified remediation potential using the BMRSI described by Robas et al. [11].

Most of the studies that have focused on the search for metallotolerant PGPR bacteria have generally been oriented toward specific bacterial genera, such as *Bacillus* [24], *Azotobacter* [25], or *Pseudomonas* [26], among others. The source of isolation is usually plants for agricultural use [27–29] since wild plants are rarely studied for isolation [19]. In the study herein, native plants from the Almadén mining district were used as a source of

isolation, thereby maximizing the probability of selecting strains with potential biotechnological use due to the selective pressure of the heavy metal and the co-evolution/coadaptation with the plant.

Of all PGPR activities, the production of auxins, which regulate cell germination and elongation, as well as root formation, is important. However, as indicated by Mirza et al. [30], the production of IAA by PGPR bacteria may vary among species and strains, as well as among conditions of cultivation, growth stage, and substrate availability. In this study, diverse concentrations of Hg might affect the growth of bacteria and the production of exogenous substances, which is something that has already been observed by Shokri and Emtiazi[31]. In their characterization of IAA-producing Gram-negative bacteria, including the genera *Agrobacterium*, *Rhizobium*, *Klebsiella*, and *Azotobacter*, they found maximum yields between 4.90 μg/mL and 5.2 μg/mL, which is a range of mean values similar to those found in this study. Surprisingly, some strains produce higher values (9, 21, 31, 37, 56, 95, and 98). However, authors of such studies do not measure IAA production in the presence of specific metal and, therefore, do not take into account the possible biochemical alterations resulting from the presence of a toxic metal, which may have a negative impact on IAA production.

Bacterial siderophores are molecules secreted in conditions of iron deficiency in order to sequester metal from their environment [32]. This paper compares the production capacity of these compounds in conditions with various concentrations of Hg, in contrast to the results obtained by Robas et al. [11], and the findings show a total inhibition of siderophore production at concentrations of 80 μg/mL and higher. Other studies [33, 34] have highlighted the importance of siderophores in protecting bacteria and plants from the hyper-accumulation of toxic metals. According to such studies, the production of some siderophores is induced by the presence of low concentrations of Hg in the medium (5μg/mL), establishing analogous competition between Fe and Hg. The results described in this paper do not rule out the possibility that the bacteria tested have not produced siderophores due to the fact that there was a maximum concentration of up to 7 μg/mL of exchangeable Hg in the edaphic medium from which they were isolated [35].

Another PGPR strategy is to decrease ethylene levels in plants. Ethylene regulates plant growth linked to abiotic stress through the activity of the enzyme ACC deaminase (ACCd), which deaminates the immediate precursor of ethylene, the ACC [36]. ACCd levels vary widely in microorganisms since their regulation can occur at the enzymatic level or according to gene expression [37]. Studies such as those by Mendoza-Hernandez et al. [38] show that certain bacteria decrease their ACCd activity when subjected to the presence of heavy metals, which could be the case of our study herein, in which there are high concentrations of Hg.

Finally, phosphate-solubilizing microorganisms facilitate the access of plant rhizospheres to these salts, which are absorbed by the plant, improving its growth and productivity [39]. In the presence of Hg, a decrease in the number of phosphate solubilizing strains can be observed. However, those strains that retain this activity are able to solubilize phosphates even with high concentrations of Hg, making them very good candidates for later use as adjuvant bioremediation PGPRs.

Authors such as Emami et al. [40] or Bomfim et al. [41], suggest that promoting successful growth must be linked to the diverse mechanisms that operate synergistically during plant development, not just to one of them. Successful remediation by the selected strains will be a result of the combined activities of the microorganism. Therefore, in this study, the quantification of the remediation potential of the strains has been used through the application of BMRSI [11].

The genera *Bacillus* and *Pseudomonas* are described as especially abundant in the composition of edaphic bacterial communities in numerous studies. This microbiota is greatly affected by seasonal factors, as well as others, since its prevalence increases in spring and autumn when the level of moisture and photosynthates is high [42–44]. Following the criteria established by Robas et al. [11], strains with BMRSI values ≥ 6.5 and

IAA production > 5.5 μg/mL. were selected, and four of the strains met the selection criteria.

Strain 9 has been identified as *Bacillus toyonenesis.* Recent studies have described the use of this bacterium as a PGPR through the production of IAA [45]. Likewise, other studies have been published, such as those of Naguib et al. [27], regarding its tolerance to Hg. This strain has one of the highest BMRSI values in the sample (7.30), making it a good candidate for further use in bioremediation.

Tolerance to Hg has also been reported with *Brevibacterium frigoritolerans* in a study of the microbial community in sediments of the Aussa River. Khezrinejad et al. [46] have proposed the use of this bacterium as a PGPR due to its strength in producing IAA. For this reason, Strain 25 is noteworthy, as it has a BMRSI of 6.54 and an IAA production capacity of 6.30 μg/mL.

In 2006, in the area of Huelva, López et al. [47] isolated five strains from wedge sole (*Dicologlossa cuneata*), which were producing disease in a human adult. Among the isolated species, a new species was found, known as *Pseudomonas baetica*. Strain 98 is also worthy of mention, as it has a high tolerance to Hg (160 μg/mL) and a high level of auxin production (6.76 μg/mL), giving it the third-highest BMRSI value in the sample. This makes it one of the best candidates to be analyzed for its capability in promoting growth in model plants.

Strain 21 was identified as *Pseudomonas moraviensis.* This species was first isolated by Tvrzova et al. [48] in an experiment involving the selective enrichment of soil with nitroaromatic compounds. Strains of this species have also been shown to have PGPR capabilities [49,50]. Strain 21 obtained the second-highest BMRSI score of the study with 7.20 points and the highest level of auxin production (7.06 μg/mL), making it one of the best candidates as well to be studied and approved for bioremediation.

5. Conclusions

1. The presence of Hg in culture mediums directly affects the capability of PGPR bacteria by decreasing their effectiveness. Such bacteria are affected in the following order, from highest to lowest affected: Siderophores > Phosphate production > ACC deaminase > IAA production.
2. The Bio-Mercury Remediation Suitability Index (BMRSI) has proven to be a useful tool for evaluating strains in an integrated way based on their PGPR capabilities in the presence of Hg. MBC (Hg) > 100 μg/mL and BMRSI ≥ 6.5 are proposed as a strain selection criterion for later bioremediation of Hg-contaminated soils.
3. Based on the criteria described, the strains *Bacillus toyonensis* (9), *Pseudomonas moraviens*is (7), *Pseudomonas baetica* (26), and *Brevibacterium frigoritolerans* (95) have been selected as good candidates for further phyto-rhizoremediation trials of Hg-contaminated soils.

Author Contributions: Conceptualization, D.G., A.P. and P.A.J.; methodology, D.G., M.R., A.P., and P.A.J.; software, D.G.; validation, D.G.; formal analysis, D.G., A.P., and P.A.J.; investigation, D.G. and M.R.; resources, A.P. and P.A.J.; data curation, A.P.; writing—original draft preparation, D.G.; writing—review and editing, D.G., M.R., A.P., and P.A.J.; visualization, D.G.; supervision, A.P., and P.A.J.; project administration, A.P.; funding acquisition, A.P., and P.A.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research has been funded by FUNDACIÓN UNIVERSITARIA SAN PABLO CEU and BANCO SANTANDER, grant number FUSP-BS-PPC01/2014.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Acknowledgments: Department of Genomics, Complutense University of Madrid; Professor Santiago Angulo of the Department of Statistics, CEU San Pablo University.

Conflicts of Interest: The authors declare no conflict of interest.

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3. Estudio biológico de promoción del crecimiento vegetal y fitoprotección frente al mercurio

La acumulación de metales pesados induce un proceso consistente en la adsorción rápida o vinculación a la superficie biológica (biosorción), seguido por un segundo paso de transporte lento e irreversible, controlado por difusión al interior de la célula (bioacumulación). En las plantas, los efectos de los metales comienzan a manifestarse en la raíz y afectan sucesivamente a los cloroplastos y las mitocondrias de las hojas, lo que altera gravemente los procesos de fotosíntesis y de respiración. En una fase más avanzada de alteración se producen intensos cambios metabólicos y de regulación celular, y ocurre finalmente el estímulo de la senescencia por acumulación crónica del metal pesado, lo que puede desencadenar la muerte de la planta.

El presente estudio se realizó sobre plántulas de *Lupinus albus* var. Orden Dorado en suelos contaminados por mercurio. Se ensayaron las cepas de manera independiente y consorciadas dos a dos. Se evaluaron las actividades promotoras del crecimiento vegetal (PGPB), la capacidad fitoprotectora con base en los marcadores de estrés oxidativo y la bioacumulación de Hg en tejido vegetal.

Se pudo observar cómo las cepas de *P. mercuritolerans* y *P. baetica* y su consorcio, así como el consorcio conformado por *P. baetica* y el *B. frigoritolerans,* promovían en crecimiento vegetal en suelos contaminados con respecto de sus controles, principalmente promoviendo el desarrollo de los parámetros aéreos correspondientes a peso aéreo y elongación del tallo, y de los parámetros radiculares correspondientes a la elongación de la raíz y el peso radicular. Así mismo, se puedo observar cómo estas *Pseudomonas* y su consorcio eran capaces de reducir los marcadores de estrés oxidativo de la planta (actividad catalasa, superóxido dismutasa, glutatión reductasa y ascorbato peroxidasa) y ejercían un efecto fitoprotector, disminuyendo la acumulación del Hg en los tejidos vegetales respecto de sus controles. También se pudo observar una correlación entre un mejor estado de salud de la planta (menores niveles de estrés y desarrollo vegetal) con una menor cantidad de Hg en planta.

Este trabajo se publicó en dos artículos, en los cuales realicé la parte experimental, análisis y procesado de datos. Del artículo publicado en *Processes* realicé la redacción correspondiente a materiales y métodos, resultados y la búsqueda bibliográfica para la introducción y discusión de los resultados. La redacción de estos dos últimos apartados la realicé en colaboración con la Dra. Marina Robas Mora, con quien comparto coautoría.

Del artículo publicado en *Frontiers in Microbiology*, realicé el todo el trabajo experimental y posterior análisis de datos y escribí el primer borrador del manuscrito, sobre el que mis directores de tesis fueron sugiriendo diversas aportaciones que incorporé en sucesivas

revisiones previo al envío a la revista. Fui el encargado del proceso de envío y comunicación con la revista durante todo el proceso de revisión y publicación.

1- Processes:
   Factor de impacto (2020): 2.847. **Categoría:** (Q3 74/143). **Fuente:** JCR (Journal of citation reports)
2- Frontiers in Microbiology:
   Factor de impacto (2020): 5.640. **Categoría:** Microbiology (Q1 28/136). **Fuente:** JCR (Journal of citation reports)

processes

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Evaluation of the PGPR Capacity of Four Bacterial Strains and Their Mixtures, Tested on *Lupinus albus* var. Dorado Seedlings, for the Bioremediation of Mercury-Polluted Soils

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Abstract: Soil contamination by mercury, which is one of the most toxic heavy metals due to its bioaccumulative capacity, poses a risk to the environment as well as health. The Almadén mining district in Ciudad Real, Spain is one of the most heavily-polluted sites in the world, making the soils unusable. Bioremediation, and more specifically phyto-rhizoremediation, based on the synergistic interaction established between plant and Plant Growth Promoting Rhizobacteria (PGPR), improves the plant's ability to grow, mobilize, accumulate, and extract contaminants from the soil. The objective of this study is to evaluate the plant growth-promoting ability of four PGPR strains (and mixtures), isolated from the bulk soil and rhizosphere of naturally grown plants in the Almadén mining district, when they are inoculated in emerged seeds of *Lupinus albus*, var. Dorado in the presence of high concentrations of mercury. After 20 days of incubation and subsequent harvesting of the seedlings, biometric measurements were carried out at the root and aerial levels. The results obtained show that the seeds treatment with PGPR strains improves plants biometry in the presence of mercury. Specifically, strain B2 (*Pseudomonas baetica*) and B1 (*Pseudomonas moraviensis*) were those that contributed the most to plant growth, both individually and as part of mixtures (CS5 and CS3). Thus, these are postulated to be good candidates for further in situ phyto-rhizoremediation tests of mercury-contaminated soils.

Keywords: PGPR; bioremediation; phyto-rhizoremediaton; mercury; soil contamination

Citation: González, D.; Blanco, C.; Probanza, A.; Jiménez, P.A.; Robas, M. Evaluation of the PGPR Capacity of Four Bacterial Strains and Their Mixtures, Tested on *Lupinus albus* var. Dorado Seedlings, for the Bioremediation of Mercury-Polluted Soils. *Processes* **2021**, *9*, 1293. https://doi.org/10.3390/pr9081293

Academic Editor: Ana Rita Lopes

Received: 15 June 2021
Accepted: 24 July 2021
Published: 26 July 2021

1. Introduction

The Almadén mining district in Ciudad Real, Spain is an area of approximately 300 km^2, which is of geological interest worldwide, due to the fact that together with the Idrija mine in Slovenia [1], it is considered to have one of the largest deposits of mercury (Hg) along with the presence of high geogenic levels of Hg. The concentrations of this heavy metal in the soils of Almadén exceed 10^6 µg/kg [2].

More than 30% of the total Hg amount that has been obtained worldwide has come from the Almadén mines [3]. The majority of this production has been extracted from the main mine in the district, the Almadén mine, whose exploitation dates back to Roman times with more than 2000 years of history. Ultimately, campaigns against the use of Hg in Europe resulted in the permanent closure of the mine's operations in 2001 and the cessation of metallurgical activity in 2003. Currently, the area is maintained as a tourist attraction [4].

Elemental Hg (Hg^0) is a heavy metal with a silvery-white color that has been used in the medical and industrial fields for many years. This metal has been used in insecticides, dyes, and protectants for wood. Currently, its use is administered at the European level by Regulation (EU) 2017/852 on mercury and, since 2013, following the legally binding UN Convention of Minamata, it has been recognized as a global pollutant [5]. Hg pollution

can come from natural sources, such as volcanic emissions, or from anthropogenic origins, such as those resulting from certain industrial processes [6]. From these emissions, Hg enters the atmosphere in the form of Hg^0 vapor, where it can remain for up to 1.7 years [7]. Through diverse geological processes, Hg is deposited in the biosphere, where it can form organic and inorganic salts. In general, these compounds tend to remain in the aqueous phase as undissociated molecules with relatively low solubility values [6]. Bearing all of this in mind, it is considered that the environmental impact of Hg is significant, since it affects surface and groundwater, air, soils, and the biosphere as well [8].

From a toxicological point of view, it is a toxic metal, or in other words, it does not have a specific biological role, which means that its incorporation into the body is not necessary. Therefore, at certain doses it produces adverse effects such as problems in the development, growth, and reproduction of living beings [9].

The cessation of mining and metallurgical activity in the Almadén mining district has had socioeconomic consequences for the population [10]. This situation has made it necessary to establish new uses for the land affected by the high concentration and provide a solution to the problem of pollution; different options have been proposed for recovering these soils. Physicochemical methods are the most widely used, though not the most favorable, due to their aggressiveness toward the environment and high costs [11]. Nowadays, from a biotechnological point of view, the most promising options for soil decontamination are bioremediation processes based on the use of living organisms (plants, fungi and bacteria) to degrade, transform or eliminate toxic compounds into harmless or less toxic metabolic products. Strategies based on the use of microorganisms and their enzymes are of increasing interest for biotechnological applications [12] due to their lower costs and lower environmental impact [13]. However, more studies are needed regarding the microbial diversity of sites contaminated with heavy metals, since those are the places where strains that are more well-adapted with greater capabilities can be identified for use in the bioremediation of these spaces.

Within the field of bioremediation study, it is worth highlighting phytoremediation, which uses the ability of plants to extract soil pollutants [14]. The effect is much stronger and more efficient when the action of the plant is combined with the activity of the bacteria present in its rhizosphere. In this sense, it is worth highlighting the so-called Plant Growth Promoting Rhizobacteria (PGPR), highly efficient to increase plant growth and increase their tolerance to biotic and abiotic factors. This particular type of phytoremediation using PGPRs in combination with plants is called phytorhizoremediation [15]. The success of this technique lies in the fact that the rhizosphere is an interface in which plants and microorganisms establish complex and varied molecular relationships, which involve the nutrients transfer, as well as specific interactions mediated by the release of signaling molecules from plant roots [16].

In the present study, as a preliminary phase prior to field trials using phytorhizoremediation in situ in the mining district of Almadén, biological tests were carried out in controlled conditions with seeds and seedlings of the *Lupinus albus* var. Orden Dorado and pre-selected isolated strains based on PGPR capabilities and their Bio-Mercury Remediation Suitability Index (BMRSI). This index provides a comprehensive assessment of the appropriateness of the strain for its successful implementation in phytorhizoremediation tests [17]. It measures the bioremediation potential of the strain by the inclusion of different PGPR activities, such as indoleacetic acid production (IAA), ACC degradation capacity (via ACC deaminase; ACCd), siderophores production (SID), ability to solubilize phosphates (PO_4^{3-}) and the maximum bactericidal concentration (MBC) of Hg, which were all measured in vitro.

The genus *Lupinus*, known as lupine, is a plant with an extracting capability for heavy metals [18]. The choice of *Lupinus albus* var. Orden Dorado was made based on its ecophysiological characteristics of strong adaptability to the conditions that occur in mines; high salinity, excessive nitrates, and a low amount of nutrients [19]. The ability to solubilize and absorb soil elements thanks to extremely robust root development makes

this plant a candidate for remediation tests in this type of environment. Studies done by Quiñones et al. in 2013 and 2018 used the rhizobacteria—*Lupinus albus* model to study the capacity of this symbiotic pair to tolerate and/or accumulate Hg. They demonstrated that the inoculation of lupine plants with Hg-tolerant strains had a clear effect on their response to exposure to this heavy metal [20,21].

This study aimed at verifying whether the inoculation of *Lupinus albus* var. Orden Dorado by PGPR strains (and their mixtures) in soils contaminated with Hg produces greater plant development, and to select the best plant growth-promoting strains for subsequent in situ tests for the phyto-rhizoremediation of soils contaminated with Hg.

2. Materials and Methods

2.1. Bacterial Strains and Mixtures Tested

The strains tested in the present study were isolated from the plant's rhizosphere and bulk soil of "Plot 6" of the mining district of Almadén in Ciudad Real, Spain [18]. The PGPR capacity in the presence of Hg of four bacterial isolates (Table 1), and six mixtures, originating from the combination of the individual isolates (Table 2), was analyzed. These four strains were selected based on their values according to the Bio-Mercury Remediation Suitability Index (BMRSI), as well as on the study of auxin production (IAA), the presence of the enzyme 1-aminocyclopropane-1-carboxylate decarboxylase (ACCd), and the production of Siderophores (SID). Phosphate solubilization was also considered in this index, although our strains did not display this capability [17]. BMRSI can be calculated by using the following formula, where the values 1 and 0 for ACCd and PO_4^{3-} indicate Presence and Absence, respectively:

$$\text{BMRSI} = [\text{IAA } (\mu g/mL) + \text{ACCd } (1/0) + \text{SID (cm)} + PO_4^{3-} (1/0)] + [\text{MBC Hg } (\mu g/mL)]$$

Table 1. Evaluation of the PGPR and BMRSI ability of the tested bacterial isolates.

Strain	[HgCl2] ppm	[IAA] ppm	ACCd (p/a)	Siderophores (cm)	BMRSI (s.u.)	Strain Origin	16s rRNA Identification
A1	140	6.29	−	2.8	9.24	*A. sativa*	*Brevibacterium frigolitolerans*
A2	140	6.16	+	0.0	7.30	SL	*Bacillus toyonensis*
B1	140	7.06	−	0.8	8.00	SL	*Pseudomonas moraviensis*
B2	160	7.85	−	0.0	8.00	*A. sativa*	*Pseudomonas baetica*

[$HgCl_2$]: maximum bactericidal Hg concentration (ppm); IAA: Indoleacetic acid production (ppm); ACCd (ACC deaminase): presence (+)/absence (−); Siderophores: measurement of the halo produced around the bacterial growth zone (cm); BMRSI (Bio-Mercury Remediation Suitability Index): unitless; Origin of the strain: SL (bulk soil), *A. sativa* (rhizosphere of *Avena sativa*).

Table 2. Mixtures formed from the strains in Table 1.

Mixtures	CS1	CS2	CS3	CS4	CS5	CS6
Strains	A1 + B1	A1 + A2	A1 + B2	B1 + A2	B1 + B2	A2 + B2

2.2. Tested Plants

Seeds of the *Lupinus albus* var. Orden Dorado from the bank of the Extremadura Scientific and Technological Research Center were used.

2.3. Substrates

Two types of substrates were used: bulk soil from "Plot 6" of the Almadén mining district and sterile vermiculite. The latter was tested to assess the effects of Hg on the tested strains, beyond the shielding effect that the soil microbial communities may provide. The characteristics of the different substrates and treatments were the following:

- Contaminated soil, high Hg concentration: sample from "Plot 6" in Almadén, specifically from "The mine on the southern slope of Cerro Buitrones" [22]. The Hg concen-

tration in this plot was 1710 mg/kg total Hg, 0.609 mg/Kg soluble Hg and 7.3 mg/Kg (8 ppm) interchangeable Hg.

- Control soil, low Hg concentration: sample from "Plot 2" in Almadén, known as Fuente del Jardinillo [18]. This plot had a concentration of 5.03 mg/kg of total Hg: 0.0417 mg/kg of soluble Hg and 0.285 mg/kg of exchangeable Hg.
- Vermiculite without Hg: vermiculite is an inert substrate with a neutral pH, which is used in hydroponic crops.
- Vermiculite with Hg: a solution of 8 ppm of Hg was added to this substrate, to recreate the growth conditions of the plant in the soil subjected to high concentrations of Hg, typical of the study area (samples from "Plot 6").

2.4. Seed Pre-Germination

As a preliminary step, the seeds were imbibed in tap water at 4 °C for 24 h. Then, they were surface sterilized with three washes with 70% ethanol for 30 s. PVC trays (40 cm × 35 cm) were used for pre-germination, filled with sterile vermiculite brought to field water capacity with sterile tap water. Subsequently, the seeds were sown and kept in the dark for 72 h at 25 ° C. Then, those seeds with an emerged radicle of 1.5 cm ± 0.2 cm were selected for further analysis.

2.5. Sowing in Different Substrates and Conditions

Sterile forest trays (Plásticos Solanas S.L., Zaragoza, Spain) were used, each composed of twelve 18-cm-high alveoli, a capacity of 300 cm^3, and a span of 5.3 cm × 5.3 cm. In total, forty-four trays were used, eleven for each type of substrate and treatment (four selected bacterial strains, their six mixtures and their respective controls without inoculum).

To avoid cross contamination, a single, pre-germinated seed was sown in each alveolus (emerged radicle approximately 3 cm). In each tray, a single strain (or mixture) and/or control was inoculated, so 12 replicates were tested for each condition. Table 3 shows the details of the loading procedure with substrates of the alveoli trays.

Table 3. Structuring of the load with substrates of each experimental group.

Type of Substrate	Content of Each Alveolus
Contaminated soil, high [Hg]	Soil of "Plot 6". Capillary irrigation with sterile water, up to field capacity.
Contaminated soil, low [Hg]	Soil of "Plot 2". Capillary irrigation with sterile water, up to field capacity.
Vermiculite with Hg	30 g of vermiculite. Capillary irrigation with 11 mL of 8 ppm $HgCl_2$ solution (2.6 mL/g), up to field capacity.
Vermiculite without [Hg]	30 g of vermiculite. Capillary irrigation, up to field capacity.

2.6. Inoculation with the Strains and Mixtures

Prior to inoculation, the strains were incubated in Nutritive Agar with 50 ppm of $HgCl_2$ for 24 h at 25 °C. Next, a Gram stain was carried out for the microscopic observation to verify the absence of contaminants. A bacterial suspension was prepared in 0.45% saline and adjusted to 0.5 McFarland (bacterial density 10^8 cfu/mL). A 0.45% saline solution was used (instead of 0.85%) to keep osmolarity. It was intended to avoid an increase in salinity, which could be aggravated by the incorporation of Hg salts and which could compromise the correct development of the seedlings. Each seed was inoculated with 1 mL of suspension.

2.7. Plant Growth Conditions

A phytotron equipped with white and yellow light was used, with a photoperiod of 11 h of light; Light intensity: 505 µmol m^{-2} s^{-1}, stable temperature at 25 ± 3 °C. Irrigation was carried out every 48 h by capillarity with sterile tap water, with a volume, experimentally, of 350 mL/tray (12 alveoli).

2.8. Harvest and Determination of Biometric Parameters

Twenty days after sowing, we proceeded to harvest (aerial and root part). To determine the biometric parameters (weight and length), the root and aerial part were washed with distilled water. The rhizospheric fraction was preserved for use in future trials. With the freshly harvested plants, the following biometric parameters were measured: total weight (g), weight of the aerial part (g), weight of the root part (g), length of the aerial part (cm), length of the root part (cm), total number of leaves, and total number of secondary roots. To study the overall behavior of each part of the plant, the previous parameters were categorized into two groups of standardized measurements: "Aerial part" and "root part", both without units. Thus, the "aerial part" includes aerial weight, aerial length, and number of leaves. The "root part" includes root weight, root length and the number of roots.

2.9. Statistical Analysis

For the statistical analysis, SPSS v.26.0 program (Version 26.0 IBM Corp, Armonk, NY, USA) was used. First, one ANOVA test was carried out to analyze the behavior of the plant in the presence of the PGPR strains in the vermiculite and soil substrates, regardless of whether they had been treated with Hg. Next, the multiple comparison test known as "Least Significant Difference" (LSD) was performed in those parameters that displayed significance (p-value ≤ 0.05). This is a post-hoc analysis using "t" tests to perform all pairwise comparisons between group means. The objective of this test is to identify the strains and mixtures that produce significant variations in any of the biometric parameters studied, compared to the controls, in the presence of the two types of substrates tested.

Next, four ANOVA tests were performed, one for each type of treatment (vermiculite with/without Hg and soil with low/high [Hg]). In the parameters in which significant differences were obtained (p-value ≤ 0.05), an LSD test was performed to identify the strains (or mixtures) that promoted significantly higher growth of *Lupinus albus*, compared to the controls, including the variable Hg.

3. Results

In order to study the influence of the substrate (soil/vermiculite), regardless of the presence of Hg on the biometric variables, a first ANOVA was performed. The ANOVA didn't show significant differences between control and plants inoculated with the different strains and mixtures when the test was performed on vermiculite. However, when tested on the soil substrate, significant differences (p-value ≤ 0.05) were obtained for the variables designated as aerial length (p-value = 0.004), aerial weight (p-value = 0.036), and aerial part (p-value = 0.010). For the latter, LSD tests were performed to detect which strains and mixtures justified the significant differences. Results are shown in Figure 1.

The obtained results show that the B2 strain produced a significantly higher increase in the aerial weight of the plants compared to control (Figure 1A). Regarding the aerial length (Figure 1B), significantly higher growth was displayed compared to control in all strains and mixtures, except for CS1 and CS2 mixtures. The most effective strain is B2, while the best mixture was CS3 (A1, *Brevibacterium frigoritolerans* + B2, *Pseudomonas baetica*).

For the aerial part (Figure 1C), all strains and mixtures with the exception of CS1 and CS2 showed significant differences in terms of general aerial growth. This growth was higher than in control plants, with B2 strain being the most significant (*Pseudomonas baetica*), as well as CS6 mixture (A2, *Bacillus toyonensis* + B2, *Pseudomonas baetica*). Leaves number on the plants did not vary significantly according to the substrate in which the plant developed.

Based on the above, B2 strain, *Pseudomonas baetica*, was the most favorable to the development of the aerial part in those plants growing in soil substrate. This strain also participated in the mixtures that contributed the most to growth: CS3 (A1, *Brevibacterium frigoritolerans* + B2, *Pseudomonas baetica*), CS5 (B1, *Pseudomonas moraviensis* + B2, *Pseudomonas baetica*) and CS6 (A2, *Bacillus toyonensis* + B2, *Pseudomonas baetica*).

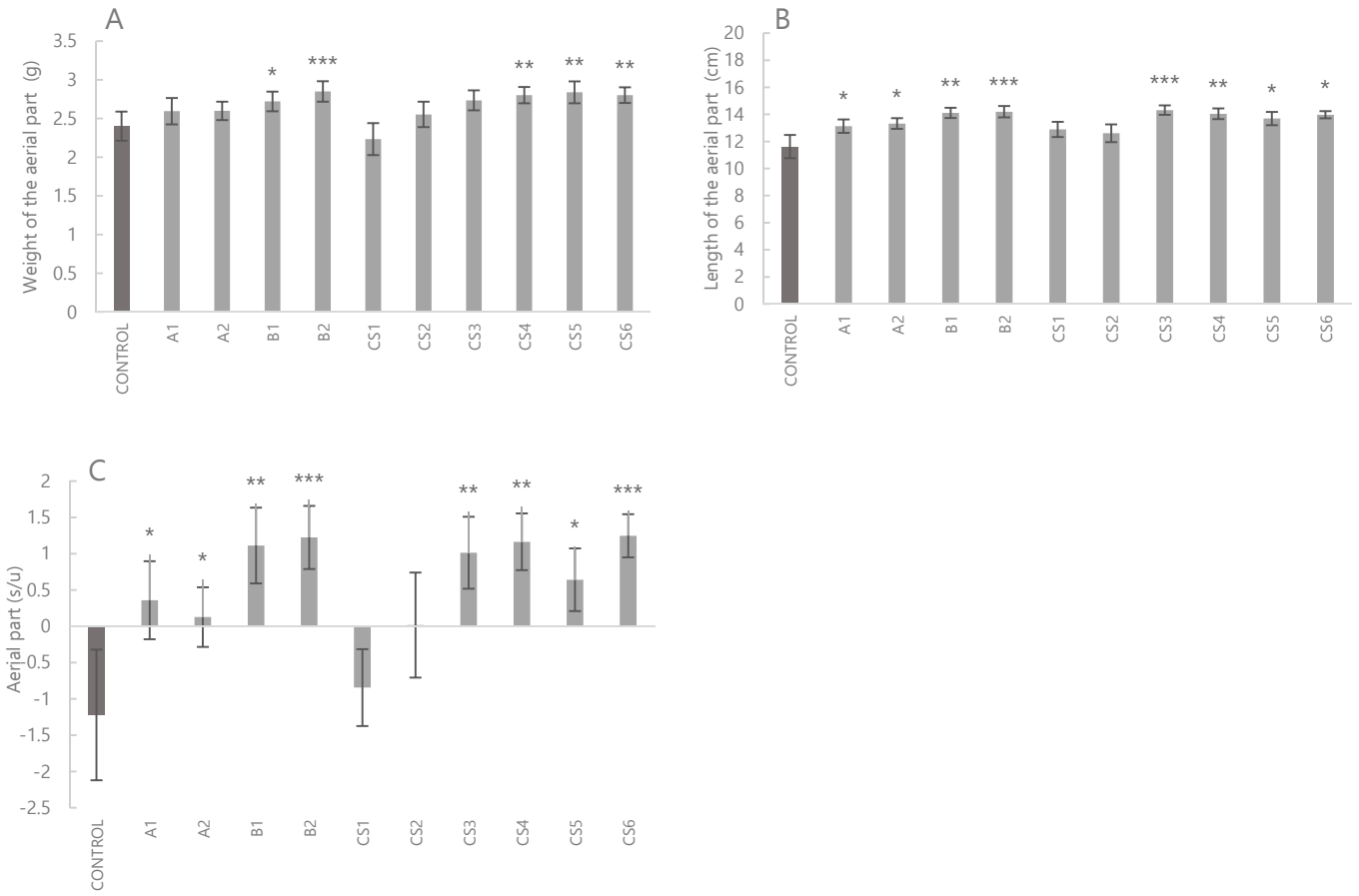

Figure 1. Results of the LSD test of the biometric parameters of plants grown in soil that showed significant differences in the ANOVA. (**A**): weight of the aerial part, (**B**): length of the aerial part, (**C**): "aerial part". The bars indicate standard error. * Indicate significant differences compared to the corresponding controls (p-value $\leq$ 0.05 and > 0.03). ** Indicate significant differences compared to the corresponding controls (p-value $\leq$ 0.03 and > 0.01). *** Indicate significant differences compared to the corresponding controls (p-value $\leq$ 0.01). s/u: no units. A1: *Brevibacterium frigolitolerans*, A2: *Bacillus toyonensis*, B1: *Pseudomonas moraviensis*, B2: *Pseudomonas baetica.* CS1 (A1 + B1), CS2 (A1 + A2), CS3 (A1 + B2), CS4 (B1 + A2), CS5 (B1 + B2), CS6 (A2 + B2).

In the second analysis, two ANOVAs were carried out to study how the presence of Hg influenced the plants' development, in each of the substrates separately (soil with high [Hg] and low [Hg]; and vermiculite with Hg and without Hg). For vermiculite, regardless of the presence of Hg or lack thereof, no significant growth differences were obtained in any of the parameters.

In soil with low [Hg], significant differences were obtained (p-value < 0.05) in the aerial weight, aerial length, and aerial part. To ascertain which strains/mixtures were responsible for the significant differences in the diverse biometric parameters in soils with low Hg concentration, an LSD test was performed. This analysis showed that B2 strain and CS6 mixture were contributing more significantly to the aerial development of the plant compared to control, especially in the increase in the weight of the aerial part (Figure 2A) and in the parameter "aerial part" (Figure 2C).

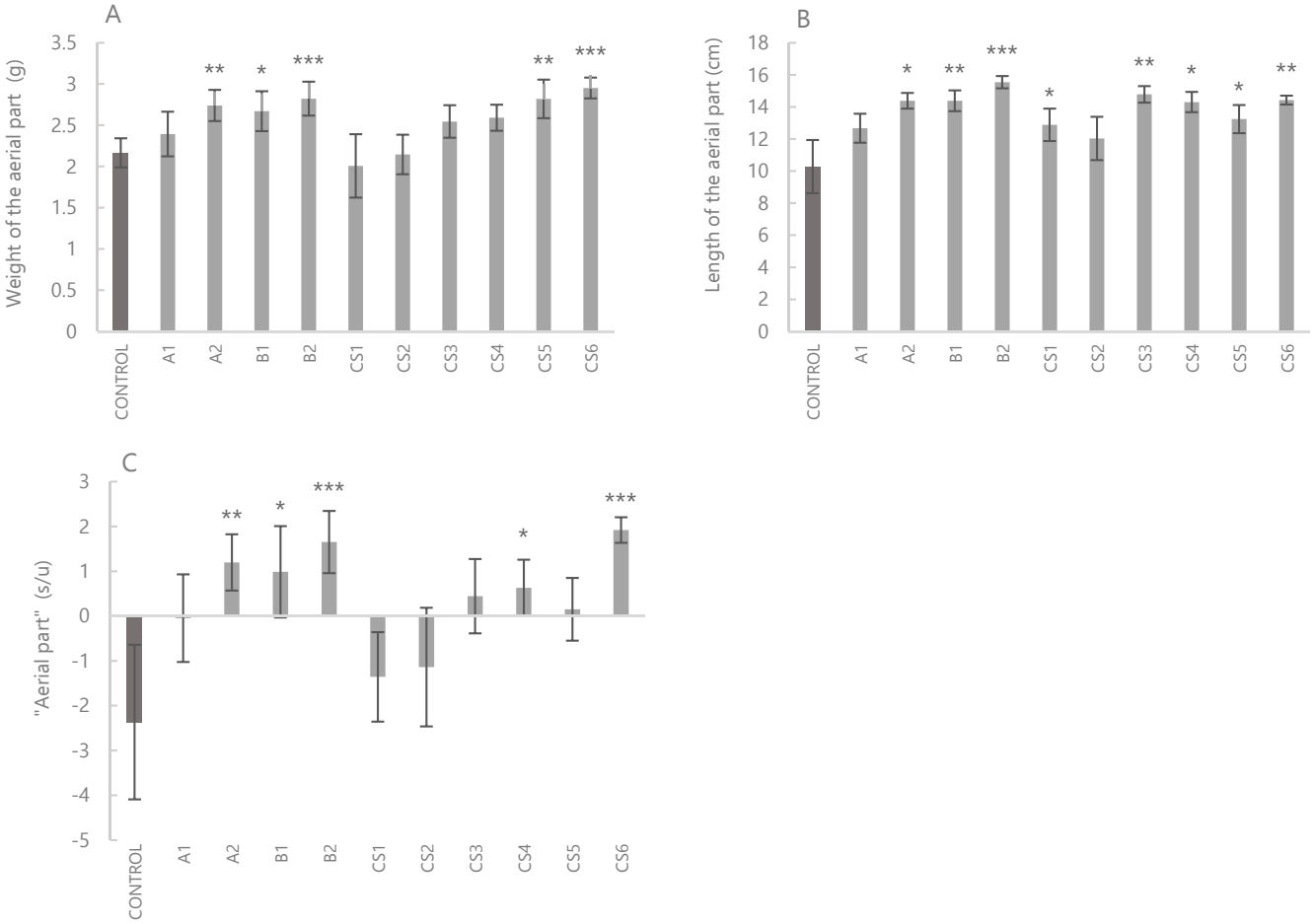

Figure 2. Results of the LSD analysis of the biometric parameters of the plants tested in low [Hg] for which significant differences were obtained based on the treatment (p-value ≤ 0.05). (**A**): weight of the aerial part, (**B**): Length of the aerial part, (**C**): "aerial part". The bars indicate standard error. * Indicate significant differences compared to the corresponding controls (p-value ≤ 0.05 and > 0.03). ** Indicate significant differences compared to the corresponding controls (p-value ≤ 0.03 and > 0.01). *** Indicate significant differences compared to the corresponding controls (p-value ≤ 0.01). s/u: no units. A1: *Brevibacterium frigolitolerans*, A2: *Bacillus toyonensis*, B1: *Pseudomonas moraviensis*, B2: *Pseudomonas baetica.* CS1 (A1 + B1), CS2 (A1 + A2), CS3 (A1 + B2), CS4 (B1 + A2), CS5 (B1 + B2), CS6 (A2 + B2).

In soil with high Hg concentration, significant differences were obtained (p-value < 0.05) for total weight, aerial part, aerial length, number of leaves, root part, root weight, and number of secondary roots (Figure 3). In this case, the CS3 mixture (A1, *Brevibacterium frigoritolerans* + B2, *Pseudomonas baetica*) is the one contributing most significantly to an increase in the weight of plants compared to controls (Figure 3A). In the root part, CS3 mixture (A1, *Brevibacterium frigoritolerans* + B2, *Pseudomonas baetica*) showed significant differences compared to controls in all biometric parameters. Noteworthy is the CS5 mixture (B1, *Pseudomonas moraviensis* + B2, *Pseudomonas baetica*), which provided greater growth in the root part parameters such as the number of roots (Figure 3–D). Strain B2 contributed stronger growth compared to control. Regarding aerial growth, the CS3 mixture (A1, *Brevibacterium frigoritolerans* + B2, *Pseudomonas baetica*) stood out once again in aerial part parameters and number of leaves (Figure 3F,G), while CS5 (B1, *Pseudomonas moraviensis* + B2, *Pseudomonas baetica*) significantly increased the growth of the aerial length compared to control (Figure 3E). Strains B1 and B2 also showed greater growth of the aerial part compared to control (Figure 3G).

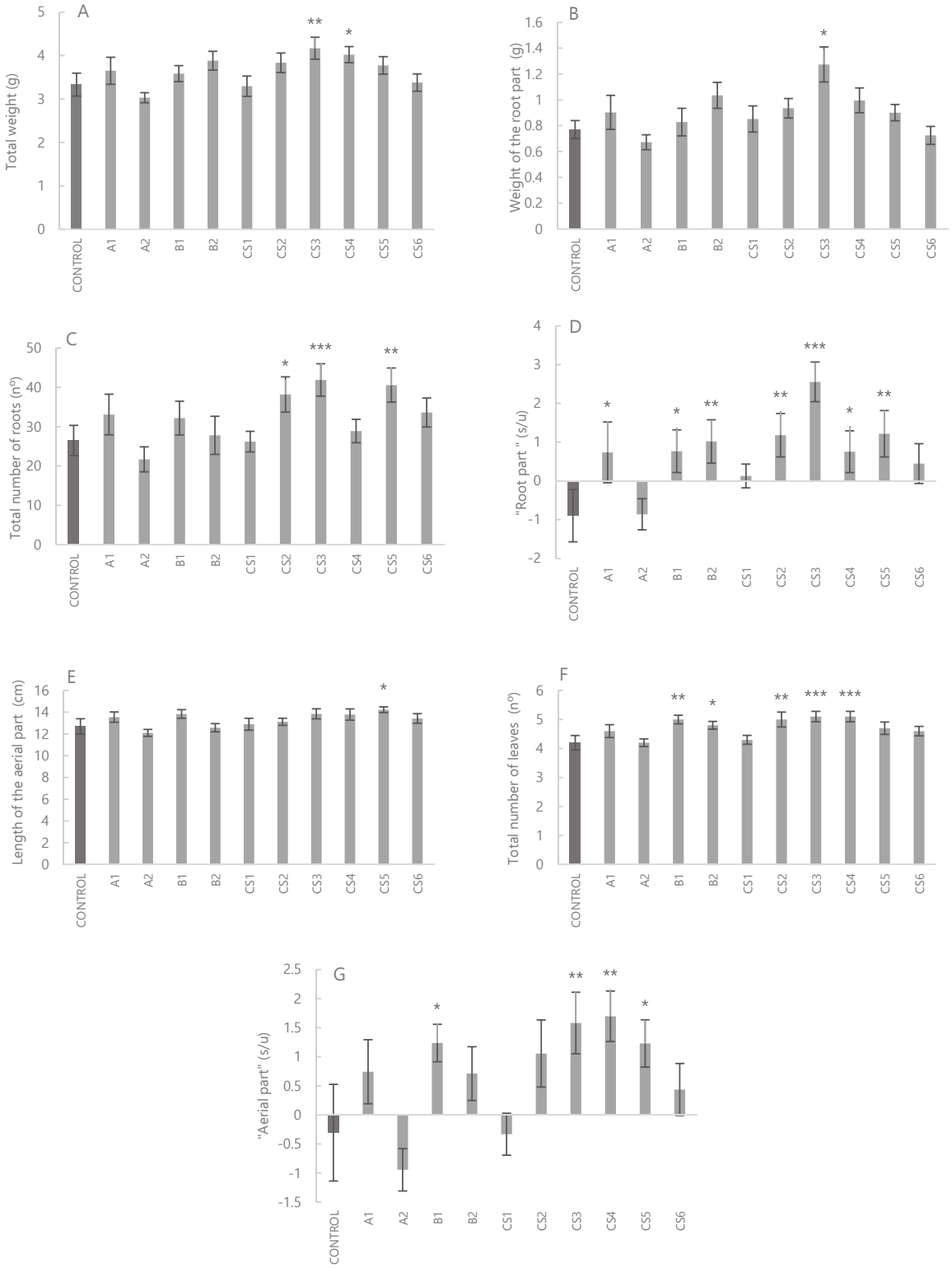

Figure 3. Results of LSD analysis of the biometric parameters of plants grown in soil with high [Hg], for which significant differences were obtained based on the treatment (p-value ≤ 0.05). (**A**): total weight, (**B**): weight of the root, (**C**): Total number of roots, (**D**): "Root part", (**E**): Length of the aerial part, (**F**): total number of leaves, (**G**): "aerial part". The bars indicate standard error. * Indicate significant differences compared to the corresponding controls (p-value ≤ 0.05 and > 0.03). ** Indicate significant differences compared to the corresponding controls (p-value ≤ 0.03 and > 0.01). *** Indicate significant differences compared to the corresponding controls (p-value ≤ 0.01). s/u: no units. A1: *Brevibacterium frigolitolerans*, A2: *Bacillus toyonensis*, B1: *Pseudomonas moraviensis*, B2: *Pseudomonas baetica.* CS1 (A1 + B1), CS2 (A1 + A2), CS3 (A1 + B2), CS4 (B1 + A2), CS5 (B1 + B2), CS6 (A2 + B2).

4. Discussion

In the present study we verified the effects on the growth of PGPR capabilities of a selection of bacterial strains (and their mixtures), in the first growth stages of *Lupinus albus* var. Orden Dorado plants by comparative analysis of their aerial biometric parameters and roots in different conditions of Hg contamination. Based on the results, a potential phytoremediation strategy based on a plant-bacteria symbiosis can be drawn up in order to offer an alternative use for soils subjected to high Hg contamination, such as those in Almadén.

Tested strains were selected based on BMRSI, which comprehensively assesses the degree of suitability of a strain for potential use in phytoremediation processes in the presence of heavy metals. In addition to the resistance to Hg, all the selected strains had PGPR capabilities, as indicated by BMRSI values greater than 6.5 [17]. PGPR strains promote plant growth on two levels: (i) directly, for example through the release of auxins or the production of ACC deaminase; and (ii) indirectly, through the synthesis of siderophores, among others. Thus, plants can develop biometrically and physiologically, even under abiotic stress by Hg thanks to the direct and indirect positive impact of PGPR. Rajkumar et al. [23] studied the same positive effect on legumes in the presence of heavy metals, once they had inoculated endophytic PGPR bacteria.

Studies have shown that the lupine *Lupinus albus* var. Orden Dorado, similar to other legumes, can extract heavy metals from soil [24], and is also tolerant to abiotic stressors, such as heavy metals presence and high salinity in soils. Therefore, it is a species widely used in bioremediation projects [25,26].

In the present study, to evaluate the effects of Hg on plant growth, the plants were grown with two different substrates (bulk soil and Vermiculite). Indeed, two types of bulk soil were used, obtained from the Almadén district (soil contaminated with Hg from "Plot 6", and a control soil with low Hg concentration, extracted from "Plot 2") [17]. The latter was used as control (instead of soil without Hg), to guarantee sample homogeneity and representativeness. To use a soil lacking Hg, a plot very far from the study site (Almadén) would have had to be sampled, which would have led to soil physicochemical changes of the soil and, therefore, to alterations in samples nature. With this assumption, it could not have been concluded whether changes in the response in the plant biometry would have been due to the conditions tested (inocula) or to the variation in soil properties. This justified the use of soil from an area that had the lowest known Hg concentration yet maintained the properties of the non-microbial fraction of the soil.

The second substrate used was vermiculite. Rodríguez et al. [27] have considered this inert substrate to be suitable for evaluating the effects of Hg on the strains (and mixtures) tested, without considering the shielding effect that soil produces, as this is a complex matrix with its own resident microbiome. Moreover, they demonstrated the ability of *Lupinus* spp to absorb and accumulate Hg in the stem when grown in vermiculite in the presence of Hg. According to López [28], even though there is no interaction between vermiculite and Hg, the root system of plants is capable of absorbing and accumulating heavy metal.

To examine and identify the strains and mixtures with the best phyto-rhizoremedial capability, a statistical analysis was carried out with regard to the way in which the diverse biometric variables studied vary in the different treatments. The first observation was that plants grew better in soil than in vermiculite, regardless of whether they had been treated with Hg or not. This may be related to the fact that as soil is a complex matrix, it allows for the contribution of nutrients and greater homeostasis, which favor plant growth. The development increased when the plants were also treated with the PGPR strains and mixtures. This finding is significant for further field trials. It suggests the existence of a high survival and colonization capacity of inoculated strains in a complex environment with a resident microbial community. Thus, it cannot be ruled out that together with the effect of the physicochemical factors inherent in the soil nature (e.g., the availability of mineral nutrients), the strains tested might have efficiently competed with the native

microorganisms. Finally, it has not been ruled out that synergistic processes might become established between the inoculated strains or mixtures and the native soil strains. However, these are only working hypotheses for a possible future experiment aimed at analyzing the survival and colonization of the root by the inocula.

Once the highest growth level of plants in soil with the presence of an inoculum was verified, the effect of Hg on plant development was analyzed. The effect of Hg on the development of the plant grown in vermiculite did not show significant differences in the biometry of the plant. Consequently, the authors did not delve deeper into this analysis.

The aerial length and aerial part parameters were significantly higher in the presence of an inoculum in the two conditions tested (in soil with low and high Hg concentration) (Figures 2 and 3E,F,G). On the other hand, plants growth in the presence of an inoculum in soil with high Hg concentration was higher compared to that of their respective controls in the root parameters (root length, root weight, and number of secondary roots). This plant organ is the main entry tissue for heavy metals in the plant, mainly by diffusion processes in the medium by means of a massive flow, and through cationic exchange. Negative charges of the rhizodermis cells interact with positive charges of heavy metals present in the soil, creating a dynamic balance that facilitates entry into the cell [29]. Among these three root parameters, the most significant growth was observed in the inoculum made with CS3 mixture (strain A1, *Brevibacterium frigotolerans* + strain B2, *Pseudomonas baetica*) (Figure 3B–D). Total weight, aerial part, and number of leaves of the plants sown in soil with a high concentration inoculated with CS3 mixture were also increased (Figure 3A,E,F).

Although the strains have individual PGPR characteristics, these can be increased or decreased when combined with mixtures. It depends on the interspecific competition, or synergy phenomena, which may result in specific behavior of the mixture exceeding what is expected from the sum of the parts, or is lower than the individual strains [30,31]. Therefore, it would be useful to study how the combination of rhizobacterial strains in the different mixtures tested can affect the different plant parameters. By analyzing the growth parameters in the presence of different inocula, it was observed that the most competitive mixture was CS3 (A1, *Brevibacterium frigotolerans* + B2, *Pseudomonas baetica*), as well as mixture CS5 (B1, *Pseudomonas moraviensis* + B2, *Pseudomonas baeitca*). These mixtures promoted significantly higher growth than control in tests carried out in soil with a high Hg concentration (Figure 3). On the other hand, the most competitive mixture in plants grown in soil with low Hg concentration was CS6, as well as CS5 to a lesser extent (Figure 2). Likewise, strains B1, *Pseudomonas moraviensis* and B2, *Pseudomonas baetica*, induced the highest growth values of all the biometric parameters, both with high and low Hg concentrations. To highlight that on an individual basis, strains B1 and B2 were those with the best PGPR capabilities and, in addition, they were part of those mixtures that proved to have higher PGPR capability in soils contaminated with Hg (CS3 and CS5).

Therefore, it could be stated that B1 and B2 strains, both individually and when forming mixtures, are the best candidates for further field phytorhizoremediation experiments on soils contaminated with Hg. Similar to the genus *Pseudomonas* in general, Strain B2, which is identified as *Pseudomonas baetica*, is characterized as a producer of auxins in concentrations greater than 5 µg/mL [32]. Auxins are plant growth regulators that are widely known to be very important, even though they were not the only ones. Strain B1, identified as *Pseudomonas moraviensis*, displays a tolerance of up to 100 µg/mg of Hg [17]. This strain stands out for production of IAA, the synthesis of siderophores, and the ability to hydrolyze ACC through the synthesis of ACC deaminase enzyme, which is related to the growth promotion of plants subject to environmental stress (pressure by Hg, for example).

Furthermore, the genus *Pseudomonas* is characterized by the production of siderophores that act as chelating agents to sequester iron (Fe). Fe is an element of the soil (generally present in its ionic form Fe^{3+}), usually scarce, and one of the main micronutrients necessary for plant development. Braud et al. [33] showed that the siderophores produced by the genus *Pseudomonas* have a greater affinity not only for Fe^{3+} sequestration, but for other minerals as well, such as Hg. In the presence of Hg in the soil, the sequestration of Fe^{3+}

by siderophores is not negatively affected and remains available to plants and can also be incorporated thanks to the action of reducing enzymes that transform it into Fe^{2+}, another form that is soluble and bioavailable for the plant [34].

Strain A1 (*Brevibacterium frigoritolerans*) is also a producer of IAA, as well as siderophores. Consistent with the results obtained, other studies have already described the ability of this species to metabolize heavy metals, as well as to tolerate high concentrations of Hg [1,35].

Finally, A2 strain (*Bacillus toyonensis*) also favored the growth of *Lupinus albus*, although to a lesser extent, compared to the rest of the strains and mixtures. According to results obtained, and consistent with previous studies [36], this strain has interesting PGPR capabilities, such as the production of siderophores and IAA that make it suitable for its evaluation in future bioremediation uses.

5. Conclusions

1. Treatment of *Lupinus albus* var. Orden Dorado with PGPR strains (and mixtures) displays greater development among those plants grown in soil subjected to stress by Hg compared to those that have not received an inoculum.
2. The BMRSI appears to be a good indicator for the selection of PGPR strains for further use in phytorhizoremediation processes. Strains B1 (*Pseudomonas moraviensis*) and B2 (*Pseudomonas baetica*), used independently, are postulated as the best candidates for further in situ assays of phytorhizoremediation processes, as they promote higher growth than controls in all biometric parameters. B2 significantly increases "root part" parameter and total number of leaves" whereas B1, in addition, significantly promotes the increase in "aerial part".
3. The most promising mixtures for further testing are CS3 (A1, *Brevibacterium frigotolerans* + B2, *Pseudomonas baetica*), as it significantly increases almost all root and shoot biometric parameters and CS5 (B1, *Pseudomonas moraviensis* + B2, *Pseudomonas baeitca*), which significantly increases the number of roots and the aerial part length.

Author Contributions: Conceptualization, M.R., A.P. and P.A.J.; methodology, M.R., A.P., C.B., D.G. and P.A.J.; software, M.R.; validation, M.R.; formal analysis, M.R., A.P. and P.A.J.; investigation, M.R. and D.G.; re-sources, A.P. and P.A.J.; data curation, A.P.; writing—original draft preparation, M.R., C.B. and D.G.; writing—review and editing, M.R., A.P. and P.A.J.; visualization, M.R.; supervision, A.P. and P.A.J.; project administration, A.P.; funding acquisition, A.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by FUNDACIÓN UNIVERSITARIA SAN PABLO CEU AND BANCO SANTANDER, grant number FUSP-BS-PPC01/2014.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Acknowledgments: Agradecemos al Centro de Investigaciones Científicas y Tecnológicas de Extremadura las facilidades ofrecidas para el acceso a las semillas de *Lupinus albus* var. Dorado.

Conflicts of Interest: The authors declare no conflict of interest.

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frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 29 September 2022
DOI 10.3389/fmicb.2022.907557

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Terrestrial Microbiology, a section of the journal Frontiers in Microbiology

RECEIVED 29 March 2022
ACCEPTED 12 September 2022
PUBLISHED 29 September 2022

CITATION
González-Reguero D, Robas-Mora M, Probanza A and Jiménez PA (2022) Evaluation of the oxidative stress alleviation in *Lupinus albus* var. orden Dorado by the inoculation of four plant growth-promoting bacteria and their mixtures in mercury-polluted soils. *Front. Microbiol.* 13:907557. doi: 10.3389/fmicb.2022.907557

Evaluation of the oxidative stress alleviation in *Lupinus albus* var. orden Dorado by the inoculation of four plant growth-promoting bacteria and their mixtures in mercury-polluted soils

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Mercury (Hg) pollution is a serious environmental and public health problem. Hg has the ability to biomagnify through the trophic chain and generate various pathologies in humans. The exposure of plants to Hg affects normal plant growth and its stress levels, producing oxidative cell damage. Root inoculation with plant growth-promoting bacteria (PGPB) can help reduce the absorption of Hg, minimizing the harmful effects of this metal in the plant. This study evaluates the phytoprotective capacity of four bacterial strains selected for their PGPB capabilities, quantified by the calculation of the biomercuroremediator suitability index (IIBMR), and their consortia, in the *Lupinus albus* var. orden Dorado. The oxidative stress modulating capacity in the inoculated plant was analyzed by measuring the activity of the enzymes catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GR). In turn, the phytoprotective capacity of these PGPBs against the bioaccumulation of Hg was studied in plants grown in soils highly contaminated by Hg vs. soils in the absence of Hg contamination. The results of the oxidative stress alleviation and Hg bioaccumulation were compared with the biometric data of *Lupinus albus* var. orden Dorado previously obtained under the same soil conditions of Hg concentration. The results show that the biological behavior of plants (biometrics, bioaccumulation of Hg, and activity of regulatory enzymes of reactive oxygen species [ROS]) is significantly improved by the inoculation of strains B1 (*Pseudomonas moraviensis*) and B2 (*Pseudomonas baetica*), as well as their corresponding consortium (CS5).

In light of the conclusions of this work, the use of these strains, as well as their consortium, is postulated as good candidates for their subsequent use in phytostimulation and phytoprotection processes in areas contaminated with Hg.

KEYWORDS

heavy metal, reactive oxygen species (ROS), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), phytoprotection

Introduction

Heavy metal pollution is an environmental threat that affects all types of living organisms, including plants, animals, and humans. Particularly, mercury (Hg) is one of the most polluting heavy metals. Even at relatively low concentrations, it has the ability to bioaccumulate and transmit through the food chain (Bjørklund et al., 2019). The accumulation of Hg can lead to pathologies that affect the central nervous system, one of the most important being Minamata syndrome (Gil-Hernández et al., 2020; Marumoto et al., 2020).

The presence of Hg at low concentrations is widely described in numerous ecosystems. Exceptionally, environments with extremely high concentrations of this heavy metal have also been described, such as those detected in the mining region of Almadén (>8889 μg/g de Hg) (US Environmental Protection Agency, 2011). The presence of this heavy metal and other polluting substances can affect plant development (Kim et al., 2017; Loix et al., 2017; Sachdev et al., 2021).

One way to evaluate the effects of Hg pollution on plant development is by studying its response to this abiotic stress. To do this, plants synthesize antioxidant enzymes that fight reactive forms of oxygen (ROS). ROS accumulation alters the metabolic balance and physiology of the plant. The main types of ROS are hydrogen peroxide (H_2O_2), hydroxyl radicals (−HO), oxygen singlet ($^1O_2$), and superoxide anion (O^-_2). Its cytoplasmic accumulation induces high oxidative stress and can produce harmful effects on the cell. To mitigate these effects, detoxifying mechanisms are expressed. However, when antioxidant processes and detoxification mechanisms are not able to eliminate excess ROS, oxidative stress harms the plant (Loix et al., 2017). It is proven that Hg induces oxidative stress causing lipid peroxidation, enzymatic inactivation, DNA and membrane damage (Cargnelutti et al., 2006; Tamizselvi and Napoleon, 2022), inhibits photosynthesis, transpiration, and nutrient transport in plants (Cargnelutti et al., 2006; Zhou et al., 2007; Ajitha et al., 2021). Its effects can even lead to the premature death of the plant (Ercal et al., 2001). The accumulation of these reactive species in cells can be reduced by activating different enzyme systems, including catalase activities (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GR) (Loix et al., 2017; Sachdev et al., 2021).

The use of plant growth-promoting bacteria (PGPB) in soils contaminated with Hg has traditionally focused on the phytoextraction of this metal, as well as on the direct promotion of plant growth (Gontia-Mishra et al., 2016; Mariano et al., 2020; González et al., 2021a). These bacteria have also been used to improve the resistance of plants against different situations of abiotic stress such as salinity or desiccation (Ansari et al., 2021; Ha-Tran et al., 2021; Khalilpour et al., 2021; Ali et al., 2022), as well as the oxidative stress produced by Hg (Cho and Park, 2000; Cargnelutti et al., 2006; Ajitha et al., 2021; Quiñones et al., 2021; Çavuşoğlu et al., 2022). To alleviate the harmful effect of pollutants, plants rely heavily on bacteria present in their rhizospheres.

The present work studies the effect of the inoculation of four PGPB strains and their combination in consortia formed by pairs, on the oxidative stress of *Lupinus albus* var. orden Dorado grown in different growing matrixes with the presence of Hg. Likewise, the phytoprotective effect of PGPBs that manifest the best results in the reduction of oxidative stress is studied. As an indicator, we use the concentration of Hg accumulated in plants. Additionally, we relate these variables to biometrics and the activity of ROS-regulating enzymes.

Materials and methods

Bacterial strains and mixtures

The isolates used in this study come from the free soil and rhizosphere of plants that grow naturally on plot 6 of the mining district of Almadén in Ciudad Real, Spain (Millán et al., 2007). The strains were selected based on their Biomercuroremediator Suitability Index values (BRMSI) (Robas et al., 2021), which evaluates PGPB activities and their tolerance to Hg. The tolerance to Hg is assessed using the minimum bactericidal concentration (MBC) and the PGP activities are as follows: production of auxin (3-indoleacetic acid: IAA), presence of the enzyme 1-animociclopropane-1-carboxylate decarboxylase

(ACCd), production of siderophores (SIDs), and the solubilizing capacity of phosphates. The BMRSI is calculated using the following formula, where 1 and 0 for the ACCd and PO_4^{-3} indicate presence or absence:

$$\text{BRMSI} = [\text{IAA } (\mu\text{g mL}^{-1}) + \text{ACCd } (1/0) + \text{SID (cm)} + \text{PO}_4^{-3}\ (1/0)] + [\text{MBC Hg } (\mu\text{g mL}^{-1})]$$

The PGPB capacity in the presence of Hg of the four bacterial isolates (**Table 1**) was analyzed by González et al. (2021b) (BRMSI **Supplementary Table 1**). The activity of the four strains was tested, as well as the combination consortium in pairs (**Table 2**).

The results of the biometrics of *Lupinus albus* var. orden Dorado inoculated with these PGPB and their respective consortia are shown in **Supplementary Table 2**. In all the experiments carried out, "control" means without inoculum.

The four bacteria isolates were subjected to the mutual compatibility test by cross streak method (**Supplementary Figure 1**) in standard method agar plates (SMA, Pronadisa®, Madrid, Spain). No inhibition was observed on the cross point in any of the combinations, which indicate the compatibility among the isolates.

Tested plants

Lupinus albus var. orden Dorado seeds were used from the seed bank of the Technological and Scientific Research Centre of Extremadura.

Growing matrixes

Four types of growing matrixes were used: to free soil from the mining district of Almadén and sterile vermiculite. The characteristics of the different growing matrixes are as follows:

- Contaminated soil, high concentration of Hg ("Soil +Hg"), from "Plot 6" of the mining district of Almadén (**Table 3**).
- Control soil with low Hg concentration ("Soil −Hg"), obtained from "Plot 2" of the mining district of Almadén. The concentration of soluble and interchangeable Hg in this plot is low enough to be considered negligible (**Table 3**).
- Vermiculite without Hg ("Vermiculite −Hg"): vermiculite is an inert substrate with neutral pH commonly used in hydroponic crops.
- Vermiculite was added with a solution of 8 mg/kg of $HgCl_2$ (concentration of Hg analogous to that found in the soluble fraction of the plot "Plot 6") ("Vermiculite +Hg").

Seed pre-germination

As a preliminary step, the seeds were soaked in water at 4°C for 24 h. The surface was sterilized with three washes of 70% ethanol for 30 s (Abdel Latef et al., 2017). Trays were used with sterile vermiculite and watered with sterile water to field capacity. The seeds were then sown and kept in darkness for 72 h at 25°C. Seeds with an emerged radicle of 3 ± 0.2 cm were selected for the study.

Sowing conditions and inoculation with the strains and mixtures

Sterile forest trays were used (Plásticos Solanas S.L., Zaragoza, España), each of them composed of 12 alveoli of 18 cm in height, with a capacity of 300 cm^3, and a light of 5.3 cm × 5.3 cm. Eleven trays were used for each type of growing matrix. To avoid cross-contamination, four pre-germinated seeds were sown in each alveolus. In each tray, a single bacterial strain (or consortium) and/or control was inoculated, in such a way that 48 seeds were tested for each condition.

A bacterial suspension in 0.45% saline was performed and the inoculum density was adjusted to 0.5 McFarland. Each seed was inoculated with 1 ml of the suspension. To the control, seeds were added to 1 ml of 0.45% saline per seed without bacterial suspension.

Plant growth conditions

A plant growth chamber (phytotron) equipped with white and yellow light with a photoperiod of 11 h of light was used (light intensity: 505 μmol m^{-2} s^{-1}, temperature stable at 25 ± 3°C). Irrigation was carried out every 48 h by capillarity with sterile water, with an experimental volume of 350 mL/tray (12 alveoli).

TABLE 1 Bacterial isolates according to their BMRSI in the presence of Hg (González et al., 2021b).

Strain	$HgCl_2$ tolerance(μg/mL)	BMRSI	Strain origin	16S rRNA identification
A1	140	6.54	*Avena sativa*	*Brevibacterium frigoritolerans*
A2	140	7.30	*BS*	*Bacillus toyonensis*
B1	140	7.20	*BS*	*Pseudomonas moraviensis*
B2	140	6.92	*Avena sativa*	*Pseudomonas baetica*

TABLE 2 Consortia formed to screen the strains in Table 1.

	CS1	CS2	CS3	CS4	CS5	CS6
Strains	A1 + B1	A1 + A2	A1 + B2	B1 + A2	B1 + B2	A2 + B2

TABLE 3 Hg speciation on study soils (Millán et al., 2007).

Soil	Total Hg (mg/Kg)	Soluble Hg (mg/Kg)	Exchangeable Hg (mg/Kg)
Plot 6 (Soil +Hg)	1710	0.609	7.3
Plot 2 (Soil −Hg)	5.03	0.0417	0.285

Harvest

Twenty-one days after seeding, the plants were harvested. To carry out the enzymatic measurements, six replicas were used for each treatment. Each replica was formed by a *mixture* of two plants (one plant per alveolus) until reaching 3 g. Four enzymatic measures related to protection against oxidative stress in plants were performed. The enzymatic activities tested were superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR).

To study the concentration of accumulated Hg, three replicates were taken per treatment of each growing matrix. Each sample consists of 12 plants (three plants per alveolus) up to 25 g per sample. The analysis was only carried out in those treatments with greater statistical significance.

Antioxidative defense enzymes

The enzymes were extracted at 4°C starting from 1 g of fresh sample per replica, with a mortar and using 50 mg polyvinylpolypyrrolidone (PVPP) and 10 ml of the following medium: 50 mM of K-phosphate buffer (pH 7.8) with 0.1 mM EDTA (for SOD, CAT, and APX). The same medium, supplemented with 10 Mm of β-mercaptoethanol was used for GR.

Superoxide dismutase activity

The SOD activity was measured based on the ability of SOD to inhibit the reduction of tetrazoyl nitro-blue (NBT) by photochemically generated superoxide radicals. A SOD unit is defined as the amount of enzyme needed to inhibit the NBT reduction rate by 50% at 25°C (Burd et al., 2000).

Catalase activity

The method of Aebi (1984) was carried out. H_2O_2 consumption was monitored for 1 min at 240 nm. This was carried out by mixing 50 mM potassium phosphate buffer with 10 mM of H_2O_2 and 100 μL of the extract.

Ascorbate peroxidase activity

The reaction was measured in a total volume of 1 mL that contains 80 nM of potassium phosphate buffer, 2.5 mM H_2O_2, and 1M sodium ascorbate. To determine the oxidation ratio of ascorbate, H_2O_2 was added to begin the reaction and the reduction of absorbances was measured for 1min at 290 nm (Amako et al., 1994).

Glutathione reductase activity

Glutathione reductase activity was estimated spectrophotometrically, according to the method of Carlberg and Mannervik (1985) at 25°C and 340 nm. The reaction mixture contained 50 mM of buffer Tris–$MgCl_2$, 3 mM, 1 mM of GSSG, 50 μl of enzyme, and 0.3 mM NADPH, which were added to initiate the reaction. The activity was calculated with the initial rate of the reaction and the molar extinction coefficient of NADPH (ε_{340} = 6.22 mM−1 cm^{-1}).

Analysis of Hg content in plant

The root and aerial fraction of each replica was dried in dry heat furnaces at 60°C for 24 h. It was sprayed and each fraction was digested separately in the acidic medium (HNO_3/HCl 2/0.5% weight/volume) under pressure for the determination of trace elements according to the regulations UNE-EN 13805. The digest was analyzed by mass spectrometry with inductively coupled plasma (ICP-MS).

By using a calibration curve, a relationship between the concentration of the pattern (μg L^{-1} or mg L^{-1}) and signal (ICP-MS) was established for each of the elements. The value of the element signal in the 12 samples is interpolated on the calibration line resulting in the total concentration of the element in the sample.

The values of the Hg pattern to establish the calibration line were as follows, expressed in μg/L: 0.00; 0.05; 0.10; 0.50; 1.00; 5.00; 10.00. Expression in mg kg^{-1} from μg L^{-1}:

$$Cf\left(\frac{\mu g}{Kg}\right) = X\left(\frac{\mu g}{L}\right) \cdot D \cdot \frac{V\,(mL)}{W\,(g)} \cdot 10^{-3}$$

where Cf (mg kg^{-1}) is the sample metal content, X (μg L^{-1}) corresponds to the interpolated experimental value or the experimental value extrapolated from the standard addition, D is the dilution performed for determination, dilution factor, V (mL) corresponds to the flask volume, and W(g) to the sample weight.

Statistical analysis

For statistical analysis, SPSS v.27.0 software was used (Version 27.0 IBM Corp, Armonk, NY, USA). The Kolmogorov–Smirnov test was performed to check the normality of all

variables. Subsequently, an ANOVA of a Kruskal–Wallis factor was performed. For the statistical analysis of the total Hg concentration accumulated in the plant, the normality of the sample data was verified using the Shapiro–Wilk test. An ANOVA was performed to determine the existence of significant differences (p-value $\leq$ 0.05). Next, a *post hoc* analysis of less significance differences (LSDs) was performed with the aim of evaluating whether the differences in Hg concentration in the plant are significant. "Substrate with Hg" is considered to be the joint analysis of the data of vermiculite supplemented with Hg and soil with a high concentration of Hg. The joint analysis of the data for vermiculite without Hg and soil without Hg is considered "substrate without Hg."

A principal component analysis (PCA) was performed starting with the 3D projection of the load factors. Next, an analysis was elaborated with the biometric data (**Supplementary Table 2**; González et al., 2021a), the concentration of Hg in the plant, and the results of the ROS enzymatic activity. All the statistical differences refer to the comparison of the variables that the plants manifest according to their inocula against their respective non-inoculated controls.

Results

Antioxidative defense enzymes analysis

Kruskal–Wallis ANOVA revealed that plants grown with the different inocula in the substrates without Hg showed no significant differences in the enzyme activity produced in response to oxidative stress. In contrast, in plants inoculated with strains B1 (*Pseudomonas moraviensis*) and B2 (*Pseudomonas baetica*), as well as their respective CS5 consortium, the differences in the activity of the four enzymes were significantly lower (p-value $\leq$ 0.001) when they were grown in soils with high levels of Hg.

Figure 1 shows the Kruskal–Wallis analysis and the comparison of means of the enzymes CAT (**Figure 1A**), SOD (**Figure 1B**), APX (**Figure 1C**), and GR (**Figure 1D**). **Figures 1A–D** shows the behavior of the activity of ROS-regulating enzymes of strains B1, B2, and their respective consortium (CS5). The CS6 consortium (formed by strains A2 and B2) is able to induce a significant reduction in the activity of the SOD enzyme by jointly analyzing substrates with high Hg concentration (**Figure 1B**).

Figure 2 shows the results of the enzymatic activities of plants subjected to different bacterial inoculums, comparing the behavior in the presence of Hg vs. the absence of Hg. We can observe that the reduction of the activity of the four enzymes in plants inoculated with strains B1 and B2 in soils with Hg reduces their activity to levels similar to those observed in plants grown in substrates in the absence of Hg.

Analysis of Hg content in plant

In order to understand the bioaccumulation of Hg, we proceeded to analyze the samples of plants grown in soils with Hg whose inoculation induced a significantly lower enzymatic activity. Likewise, the data of plants inoculated with the same PGPB and grown in soils in the absence of Hg are collected comparatively (**Table 4**). The ability of *Lupinus albus to* bioaccumulate Hg is observed mainly at the root. In plants inoculated with B1 and the CS5 consortium (B1 + B2), a significant difference in the concentration of Hg in the whole plant (total, aerial, and root) is detected with respect to the control. In the aerial part of the plants subjected to the three treatments, a significant difference in the concentration of Hg with respect to the control in soils with a high concentration of Hg is also observed.

Principal component analysis

In order to discriminate the overall behavior of the plants tested on different growing matrixes with their respective inoculum, a PCA was carried out. **Figure 3** shows the 2D graphs of the load factors on a rotated space of PCA1 vs. PCA2 (**Figure 3A**), and PCA1 vs. PCA3 (**Figure 3B**). The variables are segregated into three groups according to their biological behavior, namely, enzymatic activity, biometrics, and bioaccumulation of Hg. **Table 5** shows that the accumulation of three factors explains the model with accumulative variance greater than 87%.

Figure 4 shows the 2D projected PCA model. It can be observed how the inoculum of bacteria B1 and B2 individually is segregated from the rest of the treatments. This separation corresponds to a greater effect on the decrease in enzymatic activity (ROS), as well as an increase in biometric factors. The main factor in the abscissa axis that determines the behavior of the plant turns out to be the concentration of Hg in the soil. Likewise, the main segregation factor in the ordinate axis is the treatment with an individual inoculum of PGPB B1 and B2. The phytoprotective and plant growth-promoting effects are significantly favorable in plants grown in soils with Hg when inoculated with strains B1 and B2 independently.

Discussion

In the present study, four strains have been used whose PGP activities were tested in media with the presence of Hg vs. the absence of Hg. In the same way, their respective consortia were tested in pairs (González et al., 2021b).

The plant model of (*Lupinus albus*), as well as other legumes (Harzalli Jebara et al., 2017), has phytoextractor capacity (Zornoza et al., 2010; Rocio et al., 2013; Quiñones et al., 2021).

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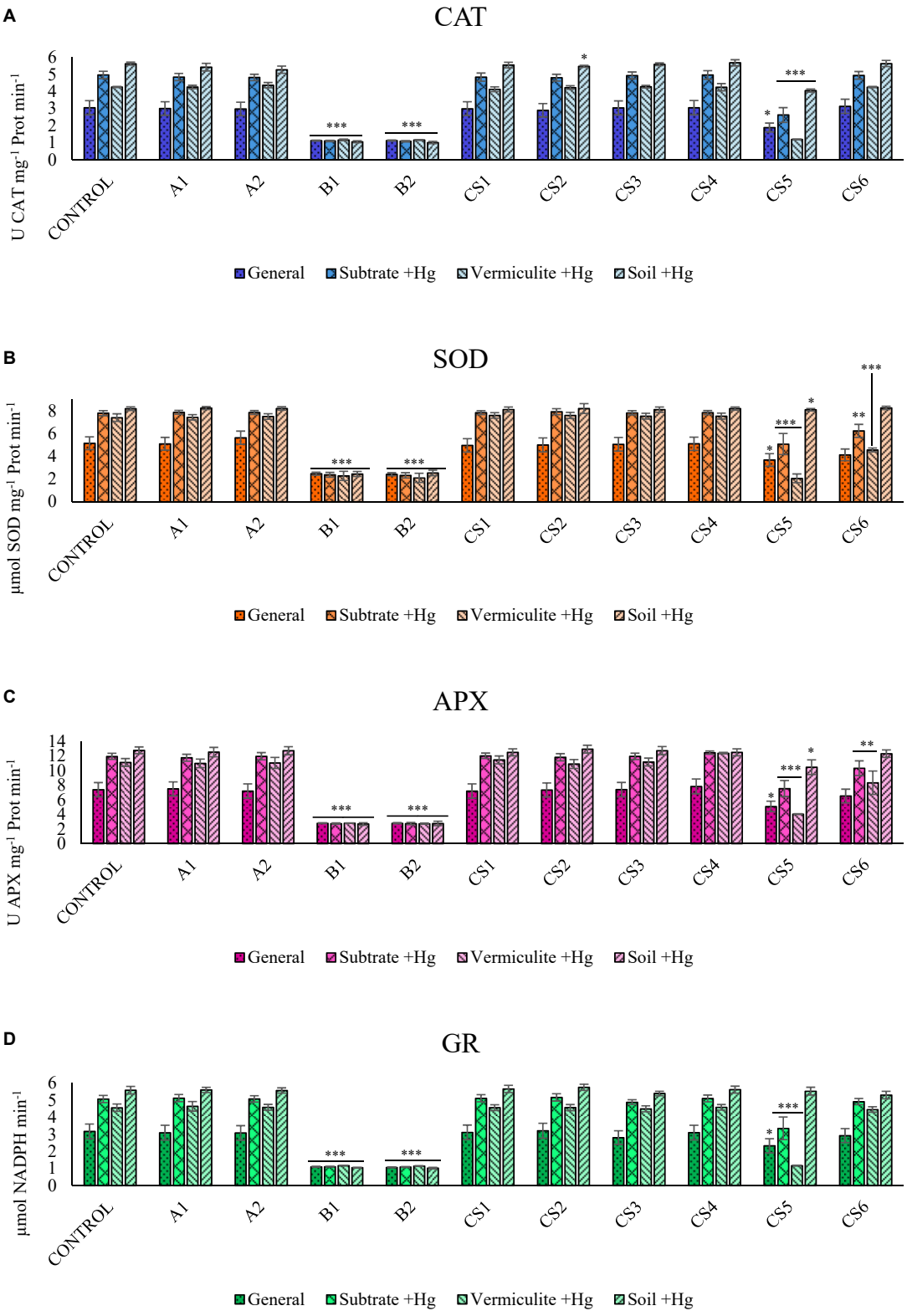

FIGURE 1
Kruskal–Wallis ANOVA results for enzyme activity: CAT **(A)**, SOD **(B)**, APX **(C)**, and GR **(D)**. Data clusters for statistical treatment: "General": dataset for plants grown in all growing matrixes; "Substrates +Hg": dataset for plants in Hg supplemented vermiculite ("Vermiculite +Hg") and soil with high Hg concentration ("Soil +Hg"); "Vermiculite +Hg": dataset for plants in supplemented vermiculite; "Soil +Hg": dataset of plants in soil with Hg high concentration. The bars indicate the standard error. Asterisks indicate the level of significance compared to control; *p-value $\leq$ 0.05, **p-value $\leq$ 0.003, and ***p-value $\leq$ 0.001.

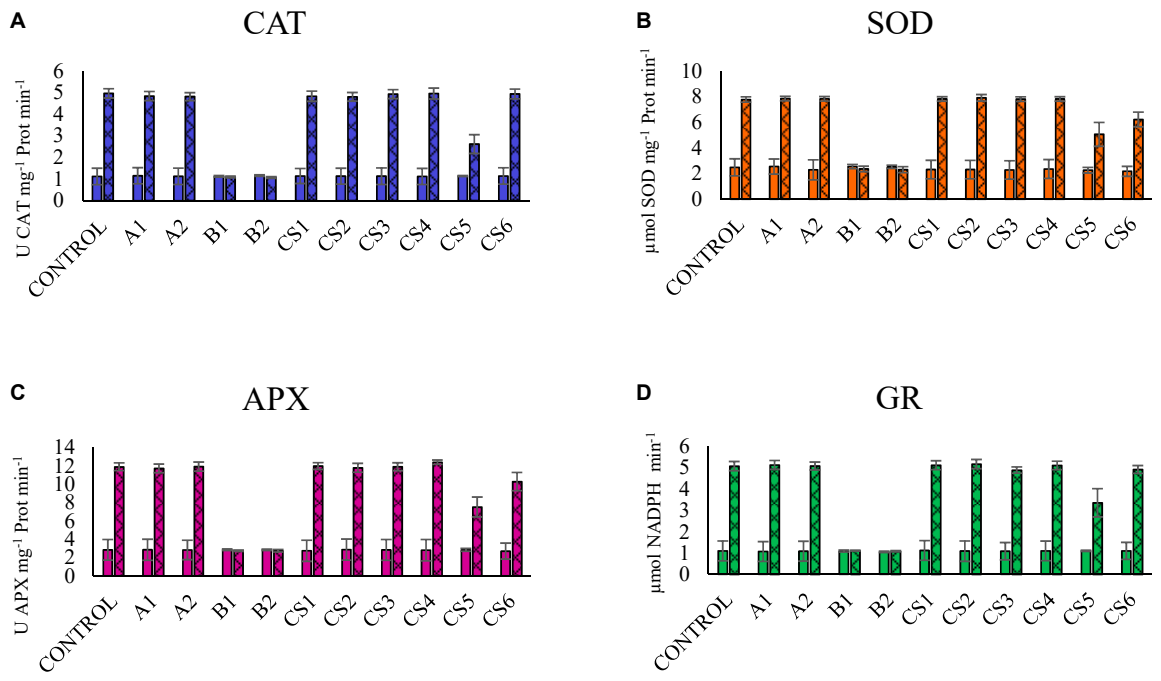

FIGURE 2
Comparison of the results of the enzymatic activity of CAT **(A)**, SOD **(B)**, APX **(C)**, and GR **(D)** in plants grown in the substrate without Hg (smooth) vs. with a high concentration of Hg (double scratching). The bars indicate the standard deviation.

In addition, its ability to absorb and resist the presence of heavy metals, such as Hg, is known. As well as its tolerance to high soil salinity (Rodriguez et al., 2007).

To evaluate the phytoprotective capacity of the strains against Hg, the plants were grown in two different substrates (free soil and vermiculite). Similarly, two types of soil were used to establish the comparison of the presence of Hg vs. the absence of Hg, both from the mining district of Almadén: soil with a high concentration of Hg (soil +Hg), and a control soil with a minimum concentration of Hg (soil −Hg). Vermiculite is a suitable substrate for the study of bacterial inocula in plants (Rodríguez et al., 2006; González et al., 2021a; Yuan et al., 2022) and avoids the shielding effect that a complex matrix, such as soil, can produce.

TABLE 4 Comparison of the concentration of Hg in the plants tested in soils with high concentration of Hg.

Treatment	Total (µg/g)	Aerial (µg/g)	Root (µg/g)
CONTROL−	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.01
B1−	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.01
B2−	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.01
CS5−	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.01
CONTROL+	10.23 ± 0.03	0.22 ± 0.02	10.01 ± 0.14
B1+	9.52 ± 0.08*	0.16 ± 0.02*	9.36 ± 0.14*
B2+	10.23 ± 0.03	0.15 ± 0.01*	10.07 ± 0.12
CS5+	7.88 ± 0.06*	0.13 ± 0.03*	7.75 ± 0.13*

*Indicates significant differences with respect to their respective controls (p-value ≤ 0.001).

Hg induces physiological and metabolic alterations in plants, such as ROS and decreased plant growth (Çavuşoğlu et al., 2022). This article analyzes the negative influence of Hg on these variables (**Figure 2**). Likewise, it is known that the use of PGPB minimizes these effects (Pirzadah et al., 2018), stimulating different defense mechanisms (Loix et al., 2017).

Antioxidative defense enzymes

Oxidative stress caused by Hg has been studied in different plant models (Cho and Park, 2000; Cargnelutti et al., 2006; Çavuşoğlu et al., 2022), observing how this heavy metal increases stress and ROS accumulation. The production of CAT, SOD, APX, and GR enzymes catalyze the degradation of H_20_2, HO^-, $^1O_2$, and O^-_2. Therefore, enzymatic activity is interpreted as a protective response against ROS, whose function is induced by the effect of Hg. The increase in CAT and SOD has been studied as a marker of oxidative stress against heavy metals in plants without a bacterial inoculum (Macar et al., 2020; Çavuşoğlu et al., 2022). In the present study, it was observed that the activity of these enzymes is significantly higher in plants grown with Hg vs. without Hg (**Figure 2**). This effect has also been observed by other authors when confronting plants with other metals, such

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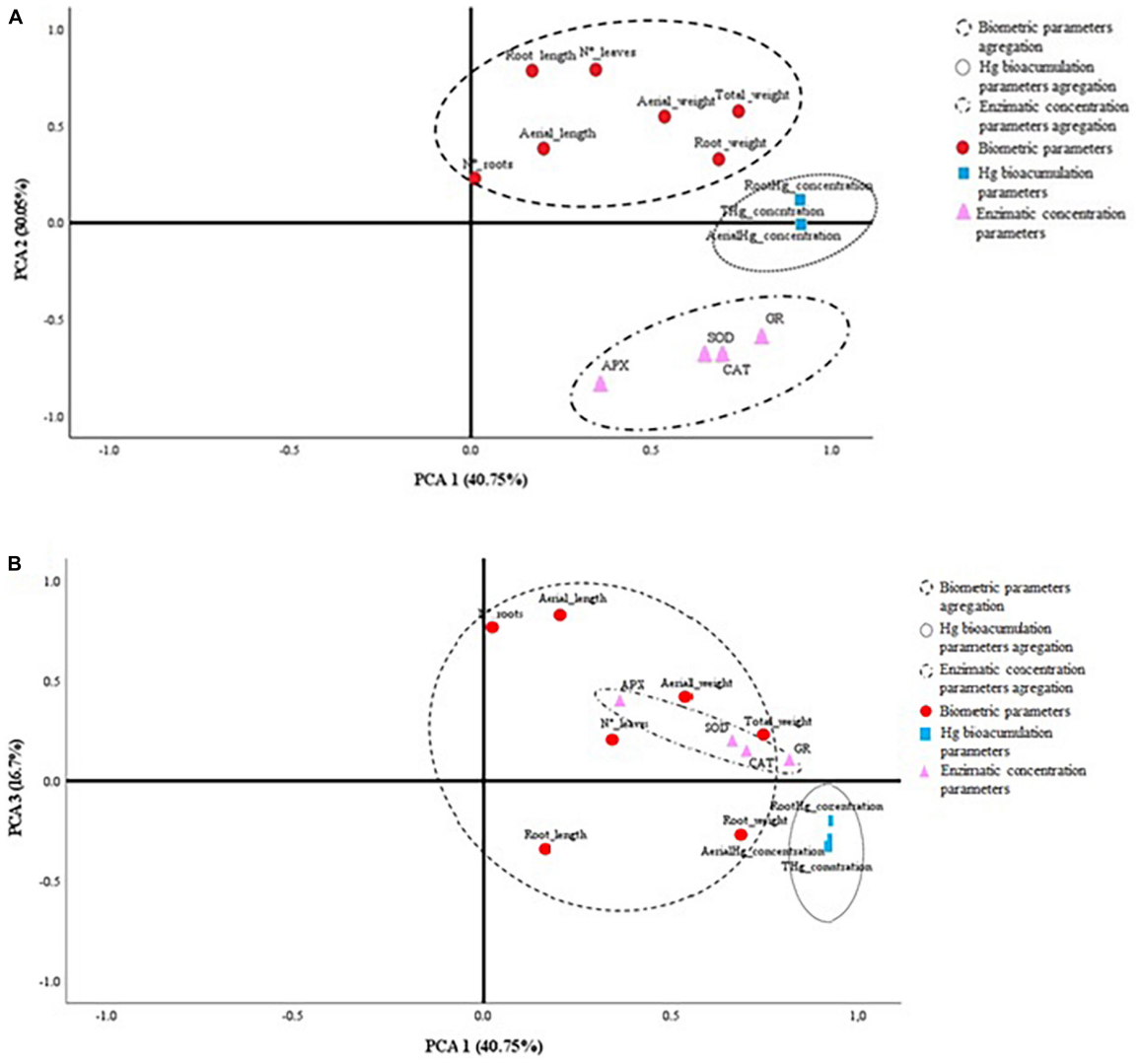

FIGURE 3
2D representation of the physiological and biometric variables of the plants according to the load factors of the PCA. **(A)** PCA1 vs. PCA2; **(B)** PCA1 vs. PCA3.

as cadmium (Cd) or lead (Pb) (Aras, 2012; Azimychetabi et al., 2021). Likewise, this effect has been observed in the enzymes APX and GR when facing different plant species with heavy metals (Hashem et al., 2016; Liu et al., 2018; Azimychetabi et al., 2021). Similarly, Pirzadah et al. (2018) investigate the effect of Hg on oxidative stress in plants not inoculated with PGPB, finding similar results to those described in the present work.

TABLE 5 Three main components that describe the model.

Component	Total	% variance	% acumulated
1	5.666	40.471	40.471
2	4.206	30.045	70.516
3	2.337	16.696	87.212

The effect that PGPB inoculation induces the decrease of ROS is known (Heidari and Golpayegani, 2012; Morcillo and Manzanera, 2021) in substrates contaminated by different heavy metals: Hg (Pirzadah et al., 2018), Pb (Abdelkrim et al., 2018), Cu (Fatnassi et al., 2015), Zn (Islam et al., 2014), and Cd (Azimychetabi et al., 2021; Renu et al., 2022). The PGPB species commonly used are those belonging to the genus *Bacillus* (Vardharajula et al., 2011; Moreno-Galván et al., 2020) and *Pseudomonas* (Sandhya et al., 2010). In the present study, the strains that produce a greater reduction in enzymatic activity in plants grown in the presence of Hg are B1 (*Pseudomonas baetica*) and B2 (*Pseudomonas moraviensis*) (**Figures 1A–D**) used both individually and in the consortium.

In the results obtained, a significant reduction in the levels of CAT (**Figure 1A**) and APX (**Figure 1C**) enzyme

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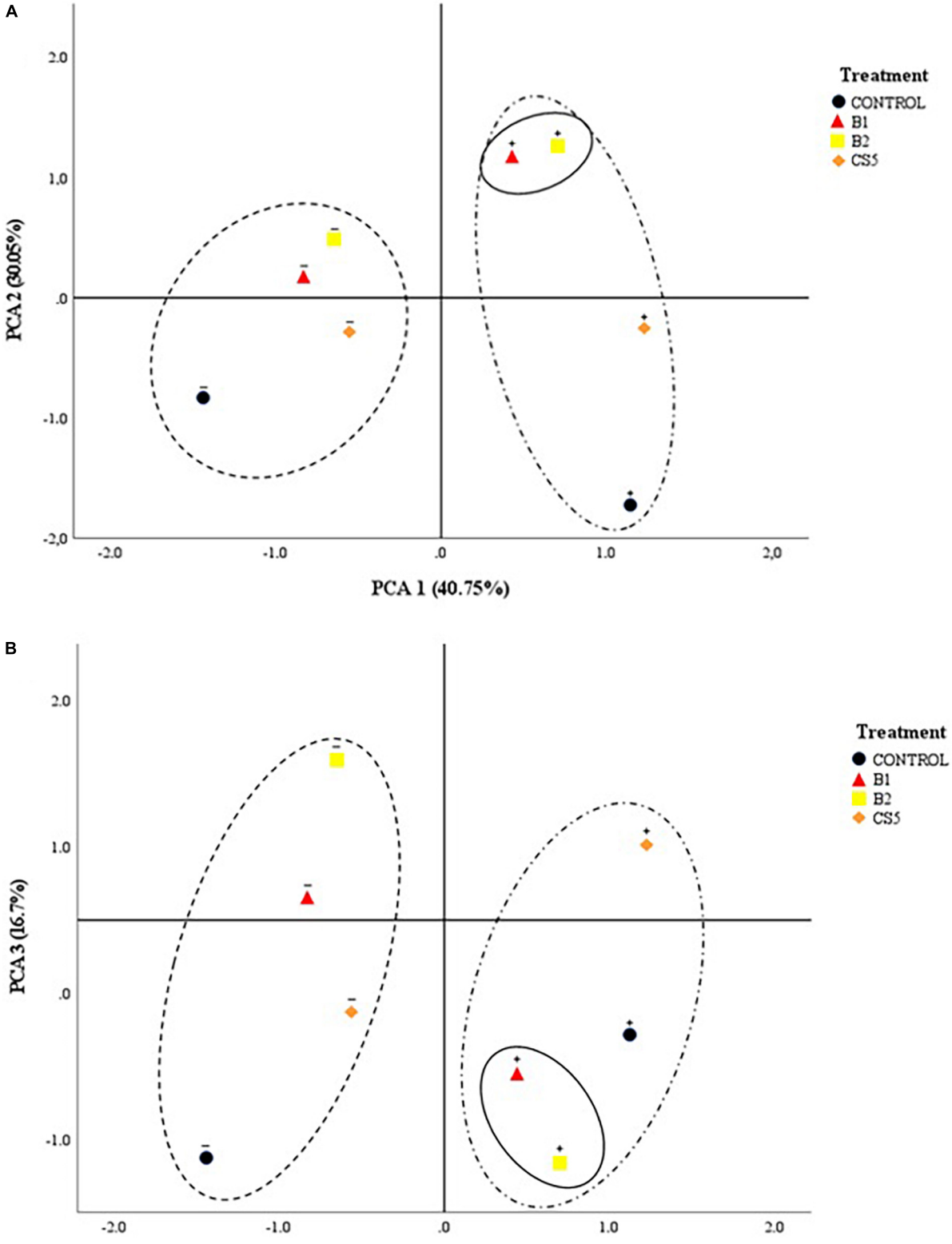

FIGURE 4
2D projection of the main components: **(A)** PCA1 vs. PCA2 and **(B)** PCA1 vs. PCA3 of the biological treatment (B1, B2, and CS5). The "+" sign on the treatment refers to "soil with a high concentration of Hg"; the "−" sign above the treatment refers to "soil without Hg."

activities was observed. This reduction is strongly correlated with the enzymatic activity of SOD (**Supplementary Table 3**). The SOD enzyme catalyzes singlet oxygen into a less reactive form of oxygen (H_2O_2). However, H_2O_2 is also toxic at high concentrations and must be eliminated by conversion to H_2O. CAT catalyzes the decomposition of H_2O_2 to H_2O and O_2. Similarly, the enzyme APX breaks down the H_2O_2 in H_2O by the reducing power of ascorbic acid. Plants possess enzymes such as CAT and APX that help maintain intracellular levels of H_2O_2 (Gill and Tuteja, 2010). For this reason, the correlation observed between the activity of SOD enzymes against CAT and APX in plants grown in the presence of Hg acquires biological meaning and is interpreted as metabolically related phytoprotection mechanisms. The inoculum of the B1 and B2 strains induce a better response of the plant subjected to oxidative stress.

Glutathione reductase is involved in the reduction of glutathione disulfide (GSSG) to glutathione (GSH) with NADPH expenditure. GSH plays a very important role in the redox regulation of the cell cycle and in the defense mechanisms against oxidative stress (Sánchez-Fernández et al., 1997). The increase in GR in substrates with Hg (**Figure 1D**) corroborates what we have found and is consistent with what has been described by other authors, indicating that how Hg increases oxidative stress in the plant (Pirzadah et al., 2018). We also observed how strains B1, B2, and their CS5 consortium (**Figure 1D**) show significantly lower enzymatic activity of this enzyme in plants grown in soil with Hg.

Analysis of Hg content in plant and principal component analysis

Plants of different species have been shown to accumulate Hg in different tissues, but the mechanism of absorption is unknown. To date, no membrane transporters involved in Hg root absorption have been identified. Due to the similarities between Cd and Hg, transmembrane Cd conveyors may be used (Lombi et al., 2001) for Hg input (Tiodar et al., 2021). The bioaccumulation of Hg in *Elodea nuttallii* has been analyzed, and it has been concluded that Cu transporters could be involved in the process (Regier et al., 2013). *Lupinus albus* is a known plant species accumulating Hg (Zornoza et al., 2010; Rocio et al., 2013; González et al., 2021a; Quiñones et al., 2021). Numerous metal carrier homologues have been identified in *Lupinus* roots (Tian et al., 2009). Whether these transporters could play a similar role in Hg absorption remains to be demonstrated. Quiñones et al. (2013, 2021) have used this plant species to demonstrate its ability to accumulate significant amounts of Hg in roots and nodules. This fact can induce a reduction in biomass production. This fact coincides with what has been observed in the present work. Nevertheless, plants inoculated with B1 and B2 are able to increase plant growth, even in substrates with high concentration of Hg. Likewise, there is evidence that inoculation with B1 and CS5 protects the plant against the contaminant, observing tissue concentrations of Hg significantly lower than the control (**Table 4**). These variables of root bioaccumulation of Hg and biometrics (total weight of the plant and root weight) present a positive correlation (**Figure 3**). In this same sense, the PCA segregates the behavior of plants treated with B1 and B2 in the presence of Hg. This fact leads us to think that the biological treatment with these strains in soils with a high concentration of Hg determines both the improvement of biometric variables, the reduction of the concentration of Hg in the plant, as well as the reduction of the activity of the enzymes that regulate the concentration of ROS.

The results of the present study show the capacity of phytoprotection against the accumulation of Hg and reduction of oxidative stress in *L. albus* var. orden Dorado of the strains B1 (*Pseudomonas moraviensis*) and B2 (*Pseudomonas baetica*), as well as of their respective CS5 consortium. For this reason, the convenience of using these strains for further use in phytostimulation and phytoprotection in soils contaminated with Hg is postulated.

Conclusion

It can be extracted as a conclusion that the biological behavior of plants [biometrics, bioaccumulation of Hg and activity of catalase enzymes (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR)] is significantly improved by inoculation with strains B1 (*Pseudomonas moraviensis*) and B2 (*Pseudomonas baetica*), as well as their corresponding consortium (CS5). In a particular way we can conclude as follows:

First, the bacteria B1 and CS5 exert a phytoprotective effect showing significantly lower systemic Hg concentration values and, especially, at the root. The B2 strain significantly reduces the bioabsorption of Hg in the aerial part of the plant.

Second, B1 and B2 significantly promote the plant growth of *Lupinus albus* growth. Its consortium (CS5) reduces oxidative stress, especially when the plant grows in highly contaminated soils with Hg.

In the light of the conclusions of this work, the use of strains B1 (*Pseudomonas moraviensis*) and B2 (*Pseudomonas baetica*) is postulated, as well as their consortium (CS5) as good candidates for their subsequent use phytostimulation and phytoprotection in areas contaminated with Hg.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding authors.

Author contributions

AP and PJ supervised the project and acquired funding for this research. All authors contributed on design the experiments, making intellectual contributions, conduct the experiments, analyze the data, writing and editing of this manuscript, and approved the submitted version.

Funding

Grant GIR: Grants to recognized research groups of the vice-rectorate of professors and researchers of the University San Pablo CEU.

Acknowledgments

We thank the Centre for Scientific and Technological Research of Extremadura for the facilities offered for access to seeds of *Lupinus albus* var. orden Dorado.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.907557/full#supplementary-material

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4. Cenoantibiograma. Estudio del perfil fenotípico de resistencias a antibióticos de una comunidad microbiológica

Los medicamentos antiinfecciosos, concretamente los antibióticos, son uno de los grupos farmacológicos más ampliamente empleados en terapéutica humana y en clínica veterinaria, como base insustituible de cualquier tratamiento antimicrobiano. Así mismo, pueden ser empleados como profilácticos en la adquisición de enfermedades transmisibles en humanos y con carácter excepcional en animales o plantas.

Los antibióticos llegan hasta los suelos por diferentes vías. Su persistencia selecciona positivamente especies bacterianas resistentes, lo que supone una pérdida de eficacia en los tratamientos farmacológicos de infecciones causadas por microorganismos por su facilidad para adquirir y portar mecanismos de resistencia con diferente grado de eficacia. Tal situación provoca una gran inquietud ante la perspectiva de que, a corto-medio plazo, la farmacología sea incapaz de hacer frente a enfermedades ocasionadas por agentes infecciosos, hasta ahora, fácilmente tratables. No obstante, por la misma razón por la que se ha constituido como principal fuente potencial para la obtención de nuevos antibióticos, también se ha convertido en un nicho de promoción y selección de resistencias bacterianas, precisamente por la amplia diversidad de microorganismos que lo habitan. El uso de microrganismos con fines biotecnológicos, ya sea para la promoción del crecimiento vegetal, biorremediación, o cualquier otro, puede generar un impacto en las comunicades que los acogen, que interesa conocer. Para ello, resulta necesario el diseño y adecuación de herramientas de control y seguimiento. Esto es necesario tanto, para comprobar si las cepas bacterianas inoculadas han tenido éxito en la colonización del medio, como para analizar su impacto sobre la comunidad previa. Habida cuenta de que los suelos contaminados pueden actuar como reservorios de resistencias a antibióticos con potencial para afectar a la salud humana, también se hace necesario la búsqueda de cepas, no sólo que produzcan un bajo impacto sobre el medio ambiente, si no que puedan ayudar como biocontrol de la aparición de potenciales nuevas cepas infectivas y/o resistencias a antibióticos de uso clínico.

El presente estudio pretende evaluar el uso del cenoantibiograma, análisis del perfil de resistencias a antibióticos de la comunidad bacteriana, como método de estudio del impacto de la introducción de inóculos bacterianos sobre comunidades microbianas edáficas. Para ello, se ensayó las variaciones del cenoantibiograma en la rizosfera de plántulas de *Lupinus albus* var. Orden Dorado, crecidas en suelos contaminados con mercurio, tras la inoculación con 4 cepas PGPB de manera independiente y consorciadas dos a dos. Del mismo modo, se realizó un análisis de amplicones de los suelos ensayados para comprender al distribución y diversidad en la comunidad edáfica previa la inoculación de los suelos. Este análisis reveló como los en los

suelos de estudio hay una gran cantidad de bacterias Gram negativas, especialmente pertenecientes a Proteobacteria, Actinobacteria y Cyanobacteria. Del mismo modo, se pudo observar como la cepa de *Brevibacterium frigoritolerans*, así como los consorcios de los que participa, son capaces de reducir el perfil de resistencias a antibióticos del suelo.

En esta publicación, realicé el todo el trabajo experimental y posterior análisis de datos. Del mismo modo, escribí el primer borrador del manuscrito sobre el que mis directores aportaron sugerencias en sucesivas rondas de revisión previo al envío a la revista. En este artículo fui el encargado del proceso de envío y comunicación con la revista durante el proceso de revisión. Este artículo se encuentra en proceso de publicación

Factor de impacto (2022): 5.168. **Categoría:** Biology (Q1 21/94). **Fuente**: JCR (Journal of citation reports)

biology

MDPI

Article

Reduction of antibiotic resistance in the rhizosphere of Lupinus albus in mercury contaminated soil mediated by the addition of PGPB

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Citation: González-Reguero, D.; Robas-Mora, M.; Probanza, A.; Jiménez, P.A. Combined use of PGPB bacteria and Lupinus albus seedlings to minimize the antibiotic resistance profile in mercury-contaminated soils. *Biology* **2023**, *12*, x. https://doi.org/10.3390/xxxxx

Academic Editor(s):

Received: date
Revised: date
Accepted: date
Published: date

Simple Summary: Mercury pollution represents a serious environmental and health problem. Additionally, it may lead to the selection of bacterial mechanisms of antibiotic resistance. The use of bacteria capable of improving plant development can help plants to better adapt to contaminated environments, to the decontamination of these sites and to prevent antibiotic-resistant bacteria from affecting animal and human health. The present study proposes a way to evaluate the beneficial effect that some bacteria can have to mitigate the spread of antibiotic resistance in mercury-contaminated soils. In the experiments carried out, it is observed how inoculated bacteria can reduce resistance to antibiotics in the soil, suggesting their potential for minimizing the dispersion of these mechanisms of antibiotic resistance.

Abstract: Mercury (Hg) pollution is a serious environmental and public health problem. Its ability to biomagnify through the trophic chain, induces numerous pathologies in humans. Likewise, the emergence of antibiotic resistance (ARs) poses a threat to the "one health" approach. The use of plant growth-promoting bacteria (PGPB) can better plant adaptation, decontamination of toxic compounds and control of AR dispersal. The cenoantibiogram, a technique that allows to know the minimum inhibitory concentration (MIC) of a microbial community, has been postulated as a tool that effectively contributes to evaluate the evolution of a soil. The present study uses the metagenomics of amplicons *16S rRNA* gene to understand the distribution of the microbial soil community prior to bacterial inoculation and cenoantibiogram technique to evaluate the ability of four PGPB and their consortia to minimize antibiotic resistance, in the rhizosphere of *Lupinus albus* var. Orden Dorado, grown in Hg contaminated soils. Results showed that the addition of A1 strain (*Brevibacterium frigoritolerans)* and its consortia with A2, B1 and B2 strains reduced the edaphic community´s MIC against cephalosporins, ertapenem and tigecycline. The metagenomic study revealed that those treatment incorporating the inoculated bacteria (*Brevibacterium*, *Pseudomonas* and *Bacillus*) had significantly increased numbers of their corresponding taxons. Additionally, the depression of microbial diversity in these treatments suggests the displacement of the autochthonous community when introducing the strains, as well as their survival capacity in the rhizosphere of *Lupinus albus*.

Keywords: Antibiotic; Biorremediation; Cenoantibiogram; Heavy metal; Mercury

1. Introduction

Mercury (Hg) is a metal with a high level of toxicity that severely affects ecosystems [1,2]. It gets incorporated and biomagnified in the food chain and, consequently, affect human health even at very low concentrations [3]. One of the areas with the highest Hg

pollution in the world is located in the mining district of Almadén (Ciudad Real, Spain), where levels of up to 8,889 μg kg^{-1} are reached [4]. In order to give alternative uses to contaminated soils, the scientific community seeks to develop actions to mitigate the effects of Hg. Several physicochemical methods have been proposed but the current trend is to use more sustainable methods, based on biotechnological techniques such as bioremediation. Therefore, there is a growing interest in the selection of microbial strains with potential bioremediation use [5]. Specifically, recent research focuses on the search for plant growth promoting (PGP) microorganisms, for their ability to promote both, plant growth and stimulate their bioremediation capabilities [5–8]. Recently, a great variety of works have been described in which methods for the selection of these microorganisms are proposed [6,9,10].

Likewise, it is known that soil microbial communities subjected to high abiotic pressure (such as heavy metal contamination), act as a natural reservoir of antibiotic resistance genes (ARG) [11,12]. Several studies describe co-selection mechanisms of various toxic compounds resistance and ARG, especially co-resistance to heavy metals and antibiotics [10,12,13]. Antibiotic resistance (AR) is an emerging global problem that has attracted the attention of the scientific community in recent years. Its development and evolution in the clinical environment is evident [14,15]. Numerous genes that enable antibiotic resistance found in pathogenic bacteria have evolved or been acquired from environmental microbial communities [16], thus the presence of multiresistant bacteria outside the hospital setting has been reported. This fact suggests the need to study and understand how the environment can behave as a reservoir of AR mechanisms. Soil is the habitat for many species that naturally produce substances with antimicrobial potential, such as *Bacillus* sp. or *Streptomyces* sp. Therefore, most AR mechanisms have an environmental origin. In nature, antibiotics can, at sub-inhibitory concentrations, exhibit different functions, such as the activation/deactivation of virulence factors or the regulation of microbial communication systems [17–20]. The positive selection of bacterial mutants in response to contaminants, such as Hg or antibiotics, could increase the mutation rate. In this way, antibiotics can act as an evolutionary force for the production and selection of new resistance mechanisms. [19]. The presence of chromosomal genes coding for resistance mechanisms explains, in part, that many bacteria, even in natural antibiotic-free environments, can naturally carry a large number of ARGs [19,21].

These ARGs can be transmitted to bacteria with clinical relevance, and new multiresistant bacteria may appear [22–25] threatening the "One Health" approach. For this reason, the World Health Organization (WHO, Geneva, Switzerland) has declared ARGs to be a new pollutant due to their emerging prevalence and wide distribution. Goal 2 of the "Global Action Plan on Antimicrobial Resistance" also sets out the need to strengthen knowledge and the scientific base on AR through monitoring and research. It highlights the importance of increasing knowledge of the emergence and spread of antibiotic resistance, among humans and animals through the environment. Besides, it also stands out the importance of developing new research tools aimed at expanding knowledge in agriculture and aquaculture, to combat the growing impact of antimicrobial resistance [26].

The expression of ARGs in different populations may be due to various factors, including the additive effect of different strains mediated by microbial communication processes such as quorum-sensing/quorum-quenching [20,27], ecological competition [28], as well as the response to abiotic factors, as in the case of the present work in which the influence of heavy metals is analyzed. Ecological competition occurs when individuals directly harm each other. In microorganisms, it refers to the secretion of metabolites that directly affect the proliferation of others, such as the secretion of antibiotics and asphyxiating polymers [28]. Co-culture experiments have shown that these secreted factors often determine which populations may prevail in complex communities [29], affecting microbial diversity [30]. Bacteria have developed methods that allow them to detect and respond directly to ecological competition by developing and selecting resistance mechanisms. In the response to biotic stress, bacteria interact with each other and regulate a set

of behaviors favorable to their survival. Many of these responses, as well as their phenotypic expression, are regulated by well-known mechanisms of microbial communication like quorum sensing/quenching mechanisms [31].

In the same way that biological competence favors the emergence and selection of AR mechanisms, several studies demonstrate the contribution of metals in the co-selection of ARGs [13,32,33]. In a strong polluted environment, the competition processes between populations favors the selection of a greater number of AR mechanisms. Likewise, other pollutants, such as antibiotics are constantly being released into the environment as a result of anthropogenic activity. This results in an antibiotic pressure for the selection of resistant strains, favoring the mobility of ARGs [34–38]. Therefore, it is of special interest to discriminate, according to the different AR mechanisms, those antibiotics that can be used as biological indicators.

Taking into account these factors, it is of special interest to search for: i) tools that allow us to perform an analysis of the resistances of a microbial community and its potential behavior as a reservoir of resistances; (ii) microbial agents capable of mitigating the effects of contaminants on soil, as well as reducing the expression of antibiotic resistance in soils.

One of the proposed tools is the so-called *cenoantibiogram*, which is defined as, the phenotypic study of antibiotic resistance of a complete microbial community [39]. The evaluation and monitoring of microbial biocenosis requires tests for environmental microbiological control. This technique is postulated as a bioindicator of the evolution of the edaphic community, as well as the comparison of different communities. In this sense, the application of the concept cenoantibiograma, opens the possibility of using a new tool to evaluate the effect of bioremediation treatments on complex bacterial communities. Likewise, the combination of this new tool with metagenomic techniques for *16S rRNA* analysis, provide an excellent opportunity to evaluate changes in the composition, diversity and structure of the soil microbial population.

The present study aims to interpret and compare the impact of the use of four PGP bacteria (PGPB) and their respective consortia on the quality and microbiological diversity of the rhizosphere of *Lupinus albus* in soils with high concentration of Hg for further environmental bioremediation assays.

2. Materials and Methods

2.1. Study area

Analyzed soils were taken from the mining district of Almadén (Ciudad Real, Spain). Specifically, the "S" slope of Cerro Buitrones on the "Plot 6", described by other authors in previous studies [40], was sampled. The concentration of Hg in this plot is 1,710 mg kg $^{-1}$ total Hg, 0.609 mg kg $^{-1}$ soluble Hg and 7.3 mg kg $^{-1}$ interchangeable Hg.

2.2. PGPB isolation, selection and characterization

The strains used for this study were isolated from bulk soil and the rhizosphere of plants naturally grown in Plot 6 of the mining district of Almadén in Ciudad Real, Spain [40]. The strains were selected based on their Biomercuroremediation Suitability Index (BRMSI) values [9], which jointly evaluates PGPB activities and their tolerance to Hg. Hg tolerance was quantified by calculating the minimum bactericidal concentration (MBC). The PGP activities analyzed were: auxin production (3-indoleacetic acid: IAA) [41], presence of the enzyme 1-animocyclopropane-1carboxylate decarboxylase (ACCd) [42], siderophores production (SID) [43] and phosphate solubilizing capacity (PO_4^{3-}) [44]. The BMRSI was calculated according to the following formula, where 1 and 0 indicate presence or absence in the variables ACCd and PO_4^{3-}:

$$\text{BRMSI} = [\text{IAA } (\mu g\ mL^{-1}) + \text{ACCd } (1/0) + \text{SID (cm)} + PO_4^{3-}\ (1/0)] + [\text{MBC Hg } (\mu g\ mL^{-1})]$$

The potential PGPB capacity in the presence of Hg of the four bacterial isolates (Table 1) was analyzed by González *et al*. [6]. For the present study, a BMRSI value > 6.5 was used as a selection criterion for the strains to be tested. For the trials, they were used individually, as well as the combination consortium in pairs, as provided in Table 2.

Table 1. Bacterial isolates according to their BMRSI in the presence of Hg [6].

Strain	$HgCl_2$ tolerance (μg mL^{-1})	BMRSI	Strain Origin	*16S rRNA* Identification
A1	140	6.54	*Avena sativa*	*Brevibacterium frigoritolerans*
A2	140	7.30	*Bulk soil*	*Bacillus toyonensis*
B1	140	7.20	*Bulk soil*	*Pseudomonas mercuritolerans*
B2	140	6.92	*Avena sativa*	*Pseudomonas baetica*

Table 2. Consortia formed by the combination of the PGPBs included in the Table 1.

	CS1	CS2	CS3	CS4	CS5	CS6
Strains	A1+B1	A1+A2	A1+B2	B1+A2	B1+B2	A2+B2

The compatibility of the consortium strains was previously checked by means of the mutual compatibility test by the cross-streak method [45] in standard method agar plates (SMA, Pronadisa®, Madrid, Spaina).

2.3. Biological assays

Seeds of *Lupinus albus* var. Orden Dorado from the seed bank of the Center for Technological and Scientific Research of Extremadura were used.

As a preliminary step, the seeds were soaked in sterile distilled water and preserved at 4 °C for 24 h. The surface was sterilized with three washes of 70 % ethanol for 30 s [46]. For pregermination, seeds were placed in trays with sterile vermiculite and irrigated with sterilized tap water until field capacity was reached. Under these conditions they were kept in darkness for 72 h at 25 °C. Seeds with an emerged radicle of 3 cm ± 0.2 cm were selected for the study.

For the biological tests, sterile forest trays were used (Plásticos Solanas S.L., Zaragoza, Spain), each of them composed of twelve alveoli 18 cm high, with a capacity of 300 cm^3, and a light of 5.3 cm × 5.3 cm. Eleven trays were used. To avoid cross-contamination, four pregerminated seeds were sown in each alveolus. A single bacterial strain (or consortium) and/or control was inoculated in each tray, so that 48 seeds were tested for each treatment.

For bacterial treatment, a bacterial suspension was performed in 0.45 % saline and the inoculum density was adjusted to 0.5 McFarland. Each seed was inoculated with 1 mL of the suspension.

A plant growth chamber (phytotron) equipped with white and yellow light with photoperiod of 11 h of light, light intensity: 505 μmol m^{-2} s^{-1}, temperature 25 ± 3 °C was used. Irrigation was carried out every 48 h by capillarity with sterile tap water, with an experimental volume of 350 mL/tray (12 alveoli).

After a growth period of 21 days, the plants were extracted from the inoculated soils by collecting the fraction of the soil intimately linked to the root. For each treatment, the rhizospheric soil of the forty-eight specimens (1-2 g per plant) was gathered and homogenized to constitute the 60 g analysis sample, which was divided into three technical replicas. For the extraction of the microbiota from each sample, the procedure described in-Velasco et al. [47] modified. To do this, 2 g of soil, of each of the technical replicas were

suspended in 20 mL of sterile saline solution (NaCl 0.45 %) and homogenized with a homogenizer Omni-Mixer at 16,000 r.p.m. for 2 minutes. It was then centrifuged at 690 g for 10 minutes with a Hettich Zentrifugen centrifuge model Mikro 22R. The remaining rhizospheric fraction (approx. 60 g per treatment) was separated into three technical replicas for metagenomic study.

2.1. DNA isolation and metagenomic analysis

The DNA was purified by the "DNeasy Power Soil Pro Kit" (Qiagen, USA) following the manufacturer's instructions. An enzyme lysis step with lysozyme was included to obtain the highest and best amount of total bacterial DNA. Purified DNA was quantified using PicoGreenTM (ThermoFisher Scientific, USA) from 40 pg. DNA isolated from each sample was used for metagenomic analysis.

Two hypervariable region (V3-V4) regions of *16S rRNA* gene were amplified using primers (341F-5'CCTACGGRRBGCASCAGGKVRVGAAT; 785R-5'GGACTAC-NVGGTWTCTAATCC). After the purification of amplicons, paired-end sequencing was done on an Illumina Mi-Seq platform. Bioinformatic analysis and quality control were performed using the Fast QC tool [48]. Q-score was used to predict the probability of an error in base-calling. Over 85% of bases >Q30 averaged across the entire run was considered acceptable. OTUs (Operative taxonomic units) were identified from all the reads using QIIME software package and a representative sequence for each OTU was also constructed.

2.1. Cenoantibiogram: AR profile of the microbial community

From the soil extract obtained in saline solution (NaCl 0.45%), it was verified that the density of viable microorganisms was $>10^8$ ufc mL^{-1} (optical density (OD) = 0.5 McFarland). It was sown in Mueller-Hinton agar (Condalab®, Madrid, Spain) and the MIC was evaluated using ε-test antibiotic strips, in triplicate, for the following antibiotics: cefuroxime, cefuroxime axetil, cefoxitin, cefotaxime, ceftazidime, cefepime, ertapenem, imipenem, amikacin, gentamicin, nalidixic acid, ciprofloxacin, tigecycline, trimethoprim/sulfamethoxazole (BioMérieux®, Marcy l'Etoile, France). Plates were then incubated according to the manufacturer's instructions. For the quantification of the MIC, the most restrictive halo was used as reference.

2.1. Statistical analysis

To evaluate the quality of the technical replicates in each soil a Pearson correlation (r) of the percent genus abundances was done. The Kolmogorov-Smirnov test was performed to check the normality of all variables. Subsequently, a one-factor ANOVA and a *post-hoc* Kruskal-Wallis analysis were performed. Similarly, a principal component analysis (PCA) was performed, starting with the 2D projection of the load factors. All statistical differences refer to the comparison of the variables manifested by plants according to their inoculum against non-inoculated soil and plant controls. SPSS program (Version 27.0 IBM Corp, Armonk, NY, USA) was used for all statistical analyses.

3. Results

3.1. Metagenomic analysis

In order to know the edaphic microbial diversity, prior to bacterial inoculation, a metagenomic analysis of amplicons of the *16S rRNA* gene was carried out to obtain the relative composition of the taxa that inhabit, both in free soil (Cont S) and in rhizospheric soil (Cont P). In order to know the edaphic microbial diversity, prior to bacterial inoculation, a metagenomic analysis of amplicons of the *16S rRNA* gene was carried out to obtain the relative composition of the taxa that inhabit, both in free soil (Cont S) and in rhizospheric soil (Cont P). In the metagenomic extraction of DNA and sequencing of the

samples, between 98% and 97% of the sequences were maintained after the QC analysis (quality control). The abundance of species between technical replications was highly correlated (all comparisons had an r > 0.9 with Pearson's correlation test). 32.7% of the readings obtained in Cont S could not be assigned to any taxon. Likewise, 16.7% could not be assigned in Cont P.

A taxonomic diversity study was conducted using the Simpson (D) and Shannon (H') diversity indices, and it can be observed that the diversity in rhizospheric soils is lower comparedto free soil (Table 3).

Table 3. Comparative table of the diversity indices of Simpson (D) and Shannon (H') for the samples studied. Where Cont S: Free control soil without plant; Cont P: Rhizospheric control soil without inoculation.

Soil	D	H′
Cont S	0.985	4.464
Cont P	0.982	3.998

The taxonomic analysis showed (Figure 1A, Table S1) a greater representation of Proteobacteria, Actinobacteria; as well as Firmicutes, Planctinomyces, Acidobacteria and Cyanobacteria in Cont S versus Cont P. It is noteworthy the low proportion of the taxonomic fraction corresponding to Firmicutes, mainly in Cont P. On the contrary, Streptomycetales and Rhizobiales appear in greater proportion in Cont P (Figure 1B, Table S2). Finally, it is worth noting the presence of sequences associated with viruses that represent similarly in both samples.

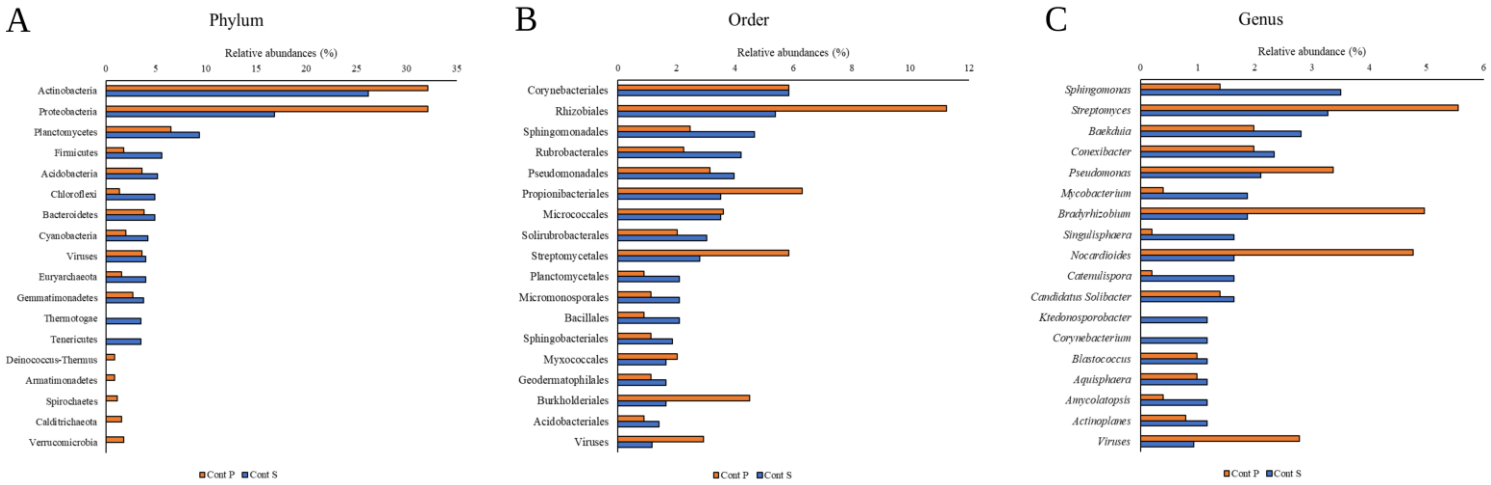

Figure 1. Relative abundances of the most representative taxa of the metagenomic analysis of amplicons of Cont S and Cont P, corresponding to the taxonomic order of Phylum (A), Order (B) and Genus (C). Relative abundance data can be seen in the Supplementary Material tables (Table S1: Phylum; Table S2: Order, and Table S3: Genus).

3.2. Antibiogram

Before the release of any biological agent into the natural environment (PGPB), its biosafety must be guaranteed (both in handling and for the health of animals and plants). To this end an antibiogram of PGPB strains was performed, which includes many of the most widespread therapeutic antibiotics (Table 4).

Table 4. MIC (μg mL^{-1}) of each of the four PGPB strains under study.

	16S rRNA identification	Cefuroxime	Cefuroxim eAxetil	Cefoxitin	Cefotaxime	Ceftacidime	Cefepime	Ertapenem	Imipenem	Amikacin	Gentamicin	Nalidixic Acid	Ciprofloxacin	Tigecyclina	Trimethoprim/Sulfomethoxazole
A1	*Brevibacterium frigoritolerans*	1	1	8	1	0.5	1	0.5	0.25	2	1	4	0.5	0.5	20
A2	*Bacillus toyonensis*	16	8	8	8	8	1	0.5	0.25	2	1	4	0.5	0.5	20
B1	*Pseudomonas mercuritolerans*	16	16	16	8	4	2	0.5	0.25	2	1	2	0.25	0.5	20
B2	*Pseudomonas baetica*	16	16	16	8	4	2	0.5	0.25	2	1	2	0.5	0.5	20

3.2. Cenoantibiogram

The cenoantibiograms of each of the treated soils was carried out. These results were then compared with the cenoantibiograms obtained from the soils without biological treatment. Table 5 shows that the soil microbial community naturally presents high MICs, to the cephalosporins antibiotic group. After the inoculation with the studied PGPB and their consortia results in a variation of the soil resistance profile This effect is especially noticeable in the case of A1 strain and its respective consortia CS1, CS2 and CS3. The main affected antibiotics regardless of the treatment are ertapenem (carbapenemmic), and tigecillin (glycylcycline). A statistically significant reduction is observed for the antibiotic cefepime between Cont S compared to biological treatments with bacterial inoculation.

Table 5. MIC (μg mL^{-1}) of the different antibiotics studied from soils inoculated with strains and/or consortia and control soils without biological treatment.

Treatment	Cefuroxime	Cefuroxime Axetil	Cefoxitin	Cefotaxime	Ceftacidime	Cefepime	Ertapenem	Imipenem	Amicacin	Gentamicin	Nalidixic Acid	Ciprofloxacin	Tigecyclina	Trimethoprime/Sulfomethoxazole
Cont S	64	64	64	16	16	4	4	0.25	2	1	8	0.5	2	20
Cont P	32	32	32	16	8	2	4	0.25	2	1	8	0.5	1	20
A1	8	8	8	1	0.5	1	0.5	0.25	2	1	4	0.5	0.5	20
A2	32	32	32	8	8	2	0.5	0.25	2	1	4	0.5	0.5	20

B1	32	32	32	16	8	2	0.5	0.25	2	1	2	0.25	0.5	20
B2	32	32	32	16	8	2	0.5	0.25	2	1	2	0.5	0.5	20
CS1	8	16	8	2	0.5	2	0.5	0.25	2	1	4	0.5	0.5	20
CS2	8	16	16	8	4	1	0.5	0.25	2	1	8	0.5	0.5	20
CS3	8	16	16	8	4	2	0.5	0.25	2	1	8	0.25	0.5	20
CS4	32	32	32	8	8	2	0.5	0.25	2	1	2	0.25	0.5	20
CS5	32	32	32	16	16	2	0.5	0.25	2	1	2	0.25	0.5	20
CS6	16	16	32	16	16	2	0.5	0.25	2	1	2	0.5	0.5	20

Table 6. ANOVA statistical analysis of a Kruskal-Wallis factor in which the MICs profile (μg mL^{-1}) of the soil is compared after being inoculated with the different bacterial strains and / or their consortia, against Cont S Cont P. "a" means a significant reduction ($p \leq 0.05$) of antibiotic concentration versus Cont S; "b" means a significant reduction ($p \leq 0.05$) of antibiotic concentration versus Cont P. Dark grey: Significant differences ($p \leq 0.05$) with Cont S and Cont P; Light gray: Significant differences ($p \leq 0.05$) with Cont P; and White: no significant differences ($p \leq 0.05$).

Treatment	Cefuroxime	Cefuroxime Axetil	Cefoxitin	Cefotaxime	Ceftazidime	Cefepime	Ertapenem	Nalidixic Acid	Ciprofloxacin	Tigecycline
Cont S	64	64	64	16	16	4	4	8	0,5	2
Cont P	32	32	32	16	8	2	4	8	0,5	1
A1	8 ^{a,b}	8 ^{a,b}	8 ^{a,b}	1 ^{a,b}	1 ^{a,b}	1 ^{a,b}	0,5 ^{a,b}	4	0,5	0,5 ^{a,b}
A2	32	32	32	8 ^{a,b}	8	2 ^{a}	0,5 ^{a,b}	4	0,5	0,5 ^{a,b}
B1	32	32	32	16	8	2 ^{a}	0,5 ^{a,b}	2 ^{a,b}	0,25 ^{a,b}	0,5 ^{a,b}
B2	32	32	32	16	8	2 ^{a}	0,5 ^{a,b}	2 ^{a,b}	0,5	0,5 ^{a,b}
CS1	8 ^{a,b}	16 ^{a,b}	8 ^{a,b}	2 ^{a,b}	1 ^{a,b}	2 ^{a}	0,5 ^{a,b}	4	0,5	0,5 ^{a,b}
CS2	8 ^{a,b}	16 ^{a,b}	16 ^{a}	8 ^{a,b}	4 ^{a}	1 ^{a,b}	0,5 ^{a,b}	8	0,5	0,5 ^{a,b}
CS3	8 ^{a}	16 ^{a,b}	16 ^{a}	8 ^{a,b}	4 ^{a}	2 ^{a}	0,5 ^{a,b}	8	0,25 ^{a,b}	0,5 ^{a,b}
CS4	32	32	32	8 ^{a,b}	8	2 ^{a}	0,5 ^{a,b}	2 ^{a,b}	0,25 ^{a,b}	0,5 ^{a,b}
CS5	32	32	32	16	16	2 ^{a}	0,5 ^{a,b}	2 ^{a,b}	0,25 ^{a,b}	0,5 ^{a,b}
CS6	16 ^{a}	16 ^{a,b}	32	16	16	2 ^{a}	0,5 ^{a,b}	2 ^{a,b}	0,5	0,5 ^{a,b}

To discriminate the overall behavior of the soils subjected to the different biological treatments, a statistical principal components analysis (PCA) was carried out. Figure 2 shows the 2D graph of the load factors. Soils treated with A1 strain (*Brevibacterium frigoritolerans)*, as well as its respective consortia CS1, CS2 and CS3, are segregated from the rest of the samples (who maintain a greater homology with the MICs of the controls).

Table 7 shows that the accumulation of two factors explains the model with a cumulative variance greater than 95%.

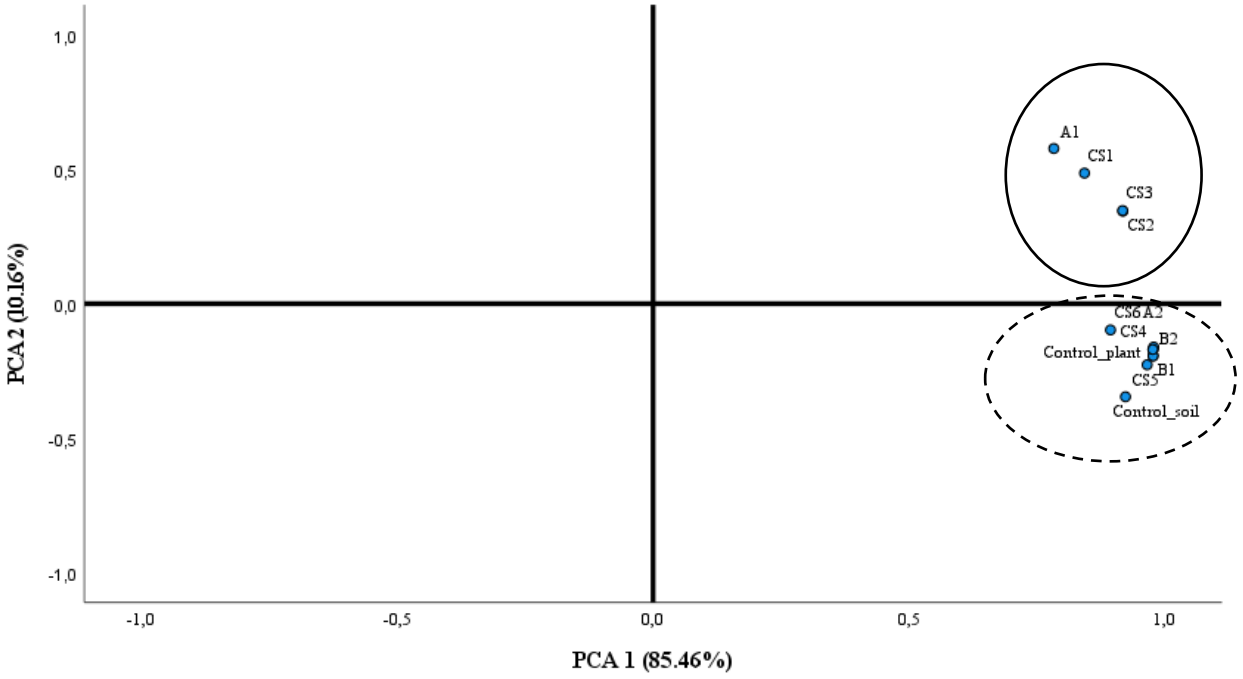

Figure 2. Representation of strains, consortia and controls according to PCA load factors (PCA1 85.46% and PCA2 10.16%).

Table 7. The two main components that describe the model.

Component	Total	% SD	% Accumulated
1	10.26	85.46	85.46
2	1.22	10.16	95.64

Figure 3 shows the PCA model ordered by antibiotics and projected in 2D. It can be observed that the data are separated into two large subsets in the abscissa axis, representing towards the positive values of this axis those antibiotics to which there is a higher MIC (A). In contrast, those antibiotics that register lower MIC values are in the negative values of this axis (B). It is interesting to note that, segregated (negative abscissa axis) are all antibiotics whose resistance is associated with point mutations or enzymes of metabolism (C). On the other hand (negative abscissa axis), we find the group of cephalosporins, whose resistance in the environment is explained by the possession of cephalosporins by microorganisms (D).

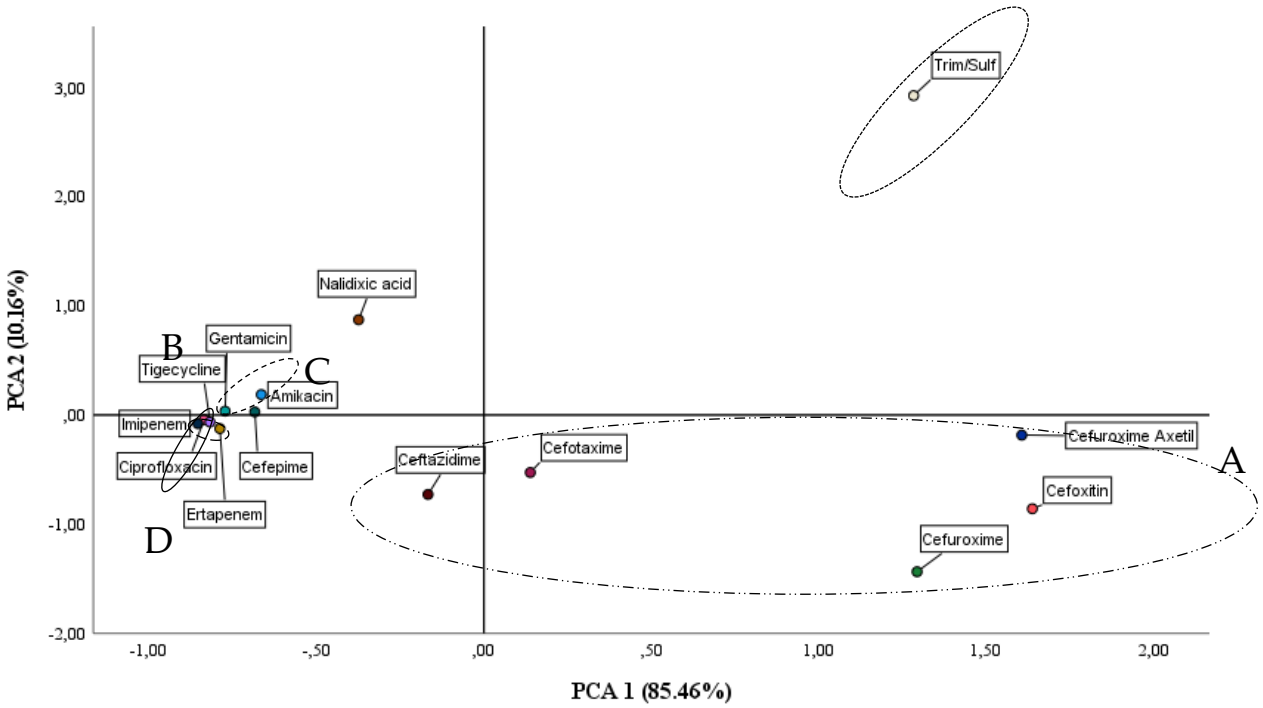

Figure 3. 2D projection of the MICs of the antibiotics studied according to the PCA load factors.

4. Discussion

There are several methods for the remediation of heavy metals contaminated soils (such as Hg). Currently, biological methods are considered more respectful towards the environment, especially those based on biotechnological techniques such as bioremediation. For this reason, there is a growing interest in the use of bioremediation, either through the use of plants, microorganisms or both (phytotorhizoremediation) for the recovery of these environments. [6,49–52]. There are multiple studies on the use of microorganisms for the recovery of heavy metal contaminated areas [8,49,50,53–56] and the benefits of adding PGPB that favor the process. Likewise, it is important to know the impact that its addition generates in the composition and biological diversity of the communities that host them, as well as the impact that the addition of microorganisms can exert on the expression of the AR mechanisms of microbial communities. The use of massive sequencing techniques can help to better interpret these results. In the present study, the analysis of *16S rRNA* was carried out at the gender level, since as several studies point out [57,58], amplicon metagenomics do not have sufficient resolution to provide reliable information at the species level. This is due to the high heterogenicity of existing and yet to be described species.

However, there is a consensus in affirming that the metagenomic analyses of the community allow a better integral interpretation of the bacterial composition of the edaphic ecosystems.

There are metagenomic and functional analyses related to the soils tested in this work [59,60] in which a high representation of Actinobacteria and Proteobacteria is described, in both soils, but with a higher abundance in Cont P (Figure 1A, Table S1). These studies also show the low representation of bacteria belonging to the phylum de Firmicutes, particularly in Cont P (Figure 1). On the contrary, we found a wide representation of the phylum Cyanobacteria in Cont S (Figure 1A, Table S1), a fact described in previous tests in these soils. The presence of this phylum has been traditionally associated in aquatic environments, but is a singularity in edaphic ecosystems, probably related to greater resistance to Hg in a similar way to other Gram-negative groups. Similarly, we

found an evident relevance of the genus *Pseudomonas* in these soils (Figure 1C, Table S3). *Pseudomonas* have the ability to adapt and integrate into a wide variety of ecosystems, being widely known their resistance to this type of media contaminated with Hg, as well as their decontamination capacity [61–64].

The results agree with the data provided by other authors in which it is shown how the taxa of Gram-negative bacteria, and especially Alpha-proteobacteria, tend to have greater representation than Gram-positive ones in these environments influenced by the presence of Hg, precisely because of their high MIC compared to said contaminate (Figure 1) [59,60].

Although the genera *Bacillus* and *Clostridium* are considered as habitual members of the edaphic communities, their abundance in the present study is very low or none, a fact already manifested in previous studies [59,60]. On the contrary, we found a strong presence of *Streptomyces,* which indicates the colonizing capacity of some Gram-positive taxa.

As can be seen in Table 3, the incorporation of exogenous biological agents such as the root of the plant, exerts a depressant effect on the soil microbiota in Cont P. Similarly, several authors describe how inoculation of a microorganism has a diversity-reducing effect. [65–68]. These characteristic changes in the distribution and activity of the microorganisms associated with the root, have been called "rhizosphere effect" [69–72]. The composition of the rhizospheric community depends directly on the root exudates, as well as on the plant species, the type of root, the age of the plant, the phenological state, as well as the type and historical use of the soil.

The results regarding the behavior of microbial communities against antibiotics coincide with those described in previous studies. In these studies, mechanisms of RA that correspond to the phenotypic profile observed in the present study are detected; particularly in the MIC against carbapenems, cephalosporins and fluoroquinolones[59,60]. In the inoculated soils there is a variation in the profile of the *cenoantibiogram*, which has as its most plausible cause a substitution in the composition and relative distribution of the original edaphic microbiota. In this same sense, other studies in which similar approaches are tested, conclude that the taxonomic groups of inoculated microorganisms significantly increase their presence in the community that hosts them [68,73].

The four bacteria used in this study have a high bioremediation potential and have been proven effective as PGPB in Hg contaminated soils (Table 1) [6,50]. PGPB capacity of *Brevibacterium frigoritolerans* strains (A1 in this study) is well described [74,75]. In some studies, strains resistant to various antibiotics have been described and may even have ARGs of high clinical relevance, such as extended-spectrum β-lactamases, cefotaxime and vancomycin resistance genes [76,77]. On the contrary, the A1 strain has a low MIC profile to most of the antibiotics tested. Although there are few references that link it as a potential infectious agent [77,78], it could it could act as a ARGs transmission agent up to clinically relevant strains. Some strains of *Bacillus toyonensis* manifest ARs of clinical relevance, such as cefotaxime, trimethoprim, ampicillin and various β-lactamases [79,80]. On the contrary, the A2 profile (tested in the present study) presents low MIC to carbapenems, aminoglycosides and fluoroquinolones (Table 4). Both *Pseudomonas mercuritolerans* (strain B1) and *Pseudomonas baetica* (strain B2) are very poorly described strains due to their recent description [62,81], in such a way that in the bibliography consulted there is no information about their AR profile. Similarly, both strains have a sensitivity profile to carbapenems, aminoglycosides and fluoroquinolones; although they could be carriers of cephalosporinases.

The absence of methods that allow global analyses of antibiotic resistance has not allowed to date to analyze precisely the impact that the addition of microorganism produces in the values of MIC compared to different antibiotics in the microbial communities that host them. The term *cenoantibiogram* refers to the phenotypic profile of resistance to different antibiotics in a community, that is, to the behavior of the population as a whole. Thus, knowing the *cenoantibiogram* of an edaphic community contributes to a more detailed knowledge of its phenotypic behavior subjected to different factors of change [39].

The addition of the A1 strain (*Brevibacterium frigoritoleras*) has the ability to significantly reduce the MIC of the bacterial community of the soils against all antibiotics tested except quinolones (nalidixic acid and ciprofloxacin). Likewise, soils inoculated with any of the 4 strains show a significant reduction in their MIC compared to ertapenem (carbapenem beta-lactam). These results show the ability of the PGPB tested to oppose biological processes such as those described by other authors [10,82–84] who claim that the genes that provide resistance to Hg and those that provide AR are co-selected, conferring antibiotic-metal co-resistance.

The same results were found in the significant reduction of MIC values compared to tigecycline (glicilcycline) of the edaphic bacterial communities when any of the strains tested are added. These results demonstrate the ability of tested PGPBs to reverse observations such as those of Rasmussen and Sørensen [85] who find that high levels of AR to tetracycline in environments with high Hg contamination could also be due to the transfer of conjugative plasmids. However, the experiment of the present work does not contradict what is collected in the literature. The phenomenon of the reduction of antibiotic resistance in a community can be explained by the displacement that the inoculated strains exert on the rest of the edaphic microorganisms, inducing functional changes that are evidenced by this decrease in MIC of the community.

Conversely, the contribution of antibiotic-resistant bacteria to the environment can induce higher MICs. This has evidenced the results of the cenoantibiograma of the analysis of different styles of cultivation and fertilization of *Vitis vinifera*, showing that those soils with a greater intervention (fertilized with fertilizer of animal origin or soils near farms) express higher MIC and greater number of resistance to antibiotics [39]. We consider, therefore, the convenience of verifying the profile of antibiotic resistance of a PGPB prior to considering its use in environmental recovery processes or agricultural or forestry exploitation. The use of the method of *cenoantibiogram* can contribute to a better understanding of the behavior of the soil, whose phenotype can be the result of different causal factors.

i. Heterogeneous composition in terms of species and strains of the community in a phenomenon of cooperative inactivation [86–88].
ii. Variability in the numerical quotas of each of the populations that make up the community [89–91].
iii. Processes of competition and/or intra- or interspecific synergy that modulate the expression of the ARGs presented by the populations [20,27].
iv. Competition between populations for environmental resources [27,31].
v. Interaction of abiotic components with the microbial community (soil environmental conditions, pH, moisture or salinity) [92–95].
vi. Interactions, synergistic or antagonistic between biomolecules that serve as mechanisms of resistance of the strains [17,19].

Both the data provided by the ANOVA of a Kruskal-Wallis factor and the PCA, show how the A1 strain and its consortia have the ability to significantly modify the *cenoantibiogram* of the soils. This postulates this strain as a very good candidate to alleviate the stress by antibiotics that a soil may suffer, with the consequent effect of alleviating the possible spread of resistance to antibiotics for therapeutic use. Similarly, the CS3 consortium, made up of strains A1 and B2, has shown in previous studies [50] its PGP capacity in *L. albus* var. Golden Order in soils highly contaminated with Hg. This bacterial consortium is able to stimulate plant growth by improving the total weight of the plant, root weight, number of roots and number of leaves. This fact, together with the results of the present study, postulate it as a good candidate for use in the bioremediation of Hg as a promoter of the reduction of MIC values.

5. Conclusions

The metagenomic analysis of soils with high concentrations of Hg shows a relative proportion of bacterial taxa belonging to Gram-negative bacteria, especially belonging to the Actinobacteria, Proteobacteria and Cyanobacteria groups. This fact could be justified by high MIC compared to the Hg of bacteria belonging to these taxa.

The addition of the A1 strain (*Brevibacterium frigoritolerans*) both isolated and consortium (CS1, CS2 and CS3) reduces the MICs to antibiotics of the edaphic community of soils contaminated with Hg. The main antibiotic groups whose MIC is significantly reduced are cephalosporins, ertapenem and tigecycline.

The CS3 consortium (*Brevibacterium frigoritoleras* + *Pseudomonas baetica*) successful in promoting plant growth in soils contaminated with Hg in previous trials, is postulated with a high bioremediator potential by significantly decreasing the values of MICs of the community that hosts them against the antibiotic's cefuroxime, cefotaxime, ertapenem and ciprofloxacin and tigecycline.

The results obtained in this study open a new horizon in the study of microbial communities through the study of the phenotypic profile of antibiotic resistance. In the same way, new paths are also opened for the study of the biosecurity of the release of microorganisms into the environment both in bioremediation processes and in the promotion of plant growth.

Supplementary Materials: The data presented in the study are deposited in the BioProyect repository, accession number for Cont_S PRJNA934906 and for Cont_P PRJNA934908. Table S1, Table S2, Table S3.

Author Contributions: Conceptualization, D.G., A.P. and P.A.J.; methodology, D.G., M.R., A.P. and P.A.J.; software, D.G. and V.F.P.; validation, D.G.; formal analysis, D.G., A.P. and P.A.J.; investigation, D.G. and M.R.; resources, A.P. and P.A.J.; data curation, A.P.; writing—original draft preparation, D.G.; writing—review and editing, D.G., M.R., A.P. and P.A.J.; visualization, D.G.; supervision, A.P. and P.A.J.; project administration, A.P.; funding acquisition, A.P. and P.A.J. All authors have read and agreed to the published version of the manuscript.

Funding: "This research was funded by Fundación Universitaria San Pablo CEU, Microbiology SAI, 259 RedLab Madrid".

Acknowledgments: Extremadura Seed Bank.

Conflicts of Interest: "The authors declare no conflict of interest."

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5. Descripción de *Pseudomonas mercuritolerans* sp. nov, y su potencial uso como PGPB en suelos contaminados con mercurio

La secuenciación del genoma completo es un método que permite una rápida anotación de genes en un organismo, y sirve como herramienta básica para posteriores análisis funcionales, tanto de los nuevos genes descubiertos, como de la propia cepa.

Una vez establecido el índice de idoneidad biomercuroremediador (IIBMR) en la aportación 2 y las capacidades testadas en plantas (en las aportaciones 3 y 4), en este estudio se planteó la secuenciación del genoma completo de las mejores cepas bacterianas con objeto de discernir su inocuidad, así como sus mecanismos moleculares de resistencia a Hg.

Los resultados de la secuenciación de dos cepas del género *Pseudomonas*, reveló que ninguna de ellas posee genes de virulencia funcionales ni mecanismos de resistencia a antibióticos transmisibles. A su vez, ambas poseen un elevado potencial genético como PGPB, siendo compatible este perfil con lo observado fenotípicamente. El bajo índice de homología con los genomas de referencia, así como los índices dDDH y ANI de la cepa identificada mediante la secuenciación del RNA 16S como "*Pseudomonas moraviensis*" han llevado a su clasificación como cepa tipo de la especie *Pseudomonas mercuritolerans* sp nov.

En el presente trabajo he participado en el análisis fenotípico de las cepas y procesado de las muestras. El análisis genético más exhaustivo, la redacción de los materiales y métodos, así como de los resultados y discusión, se ha despeñado de manera conjunta con Doña Vanesa Fernández Pastrana y la Dra. Marina Robas Mora. El proceso de comunicación con la revista, así como las revisiones y cambios durante el proceso de revisión fueron hechas por el *corresponding author* (Dra. Marina Robas Mora), con quien trabajé conjuntamente en todo momento.

Factor de impacto (2021): 6.064. **Categoría:** Microbiología (Q1 34/137). **Fuente**: JCR (Journal of citation reports)

frontiers | Frontiers in Microbiology

TYPE Original Research
PUBLISHED 06 December 2022
DOI 10.3389/fmicb.2022.1032901

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Frontiers in Microbiology Terrestrial Microbiology, a section of the journal Frontiers in Microbiology

RECEIVED 31 August 2022
ACCEPTED 14 November 2022
PUBLISHED 06 December 2022

CITATION
Robas Mora M, Fernández Pastrana VM, González Reguero D, Gutiérrez Oliva LL, Probanza Lobo A and Jiménez Gómez PA (2022) Oxidative stress protection and growth promotion activity of *Pseudomonas mercuritolerans* sp. nov., in forage plants under mercury abiotic stress conditions. *Front. Microbiol.* 13:1032901. doi: 10.3389/fmicb.2022.1032901

Oxidative stress protection and growth promotion activity of *Pseudomonas mercuritolerans* sp. nov., in forage plants under mercury abiotic stress conditions

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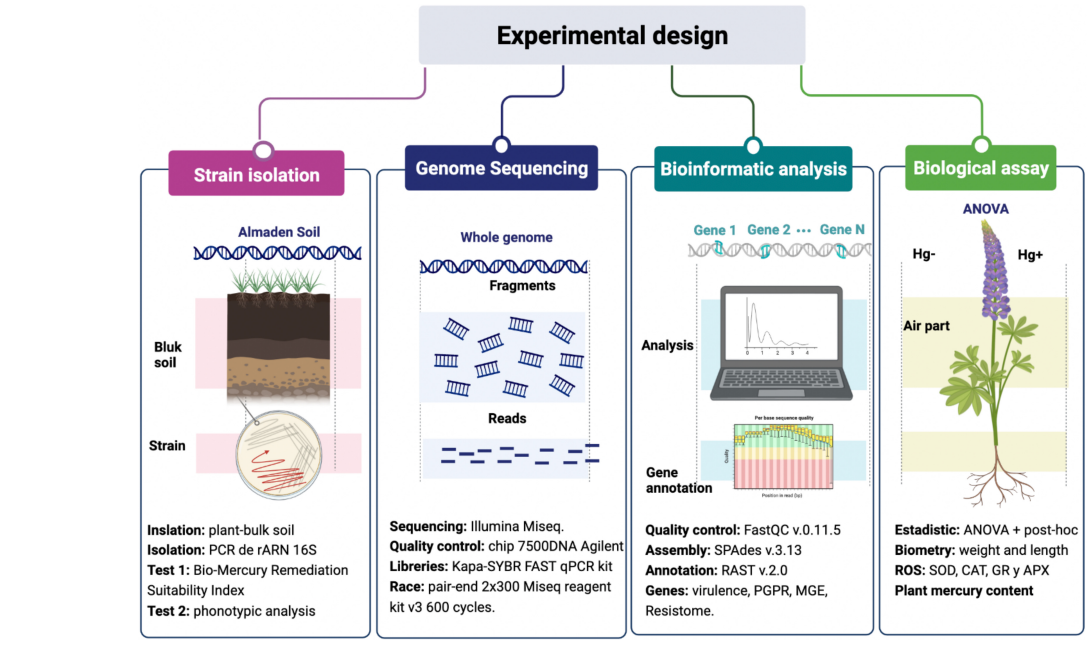
SAICEUPSM^T strain was isolated from soils in the mining district of Almadén (Ciudad Real, Spain), subjected to a high concentration of mercury. Using the plant model of lupinus, the strain was inoculated into the rhizosphere of the plant in a soil characterized by a high concentration of mercury (1,710 ppm) from an abandoned dump in the mining district of Almadén (Ciudad Real, Spain). As a control, a soil with a minimum natural concentration of mercury, from a surrounding area, was used. Under greenhouse conditions, the effect that the inoculum of the SAICEUPSM^T strain had on the antioxidant capacity of the plant was studied, through the quantification of the enzymatic activity catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), and glutathione reductase (GR). Likewise, the capacity of the plant to bioaccumulate mercury in the presence of the inoculum was studied, as well as the effect on the biometric parameters total weight (g), shoot weight (g), root weight (g), shoot length (cm), root length (cm), total number of leaves (N), and total number of secondary roots (No). Finally, in view of the results, the SAICEUPSM^T strain was identified from the phenotypic and genotypic point of view (housekeeping genes and complete genome sequencing). The inoculum with the SAICEUPSM^T strain in the presence of mercury produced a significant reduction in the enzymatic response to oxidative stress (CAT, APX, and SOD). It can be considered that the strain exerts a phytoprotective effect on the plant. This led to a significant increase in the biometric parameters total plant weight, root weight and the number of leaves under mercury stress, compared to the control without abiotic stress. When analyzing the mercury content of the plant with and without bacterial inoculum, it was found that the incorporation of the SAICEUPSM^T strain significantly reduced the uptake of mercury by the plant, while favoring its development in terms of biomass. Given the positive impact of the SAICEUPSM^T strain on the integral development of the plant,

10.3389/fmicb.2022.1032901

it was identified, proving to be a Gram negative bacillus, in vitro producer of siderophores, auxins and molecules that inhibit stress precursors. The most represented fatty acids were C16:0 (33.29%), characteristic aggregate 3 (22.80%) comprising C16:1 ω7c and C16: 1ω6c, characteristic aggregate 8 (13.66%) comprising C18:1 ω7c, and C18: 1 cycle ω6c and C 17:0 (11.42%). From the genotypic point of view, the initial identification of the strain based on the *16S rRNA* gene sequence classified it as *Pseudomonas iranensis*. However, genome-wide analysis showed that average nucleotide identity (ANI, 95.47%), DNA-DNA *in silico* hybridization (dDDH, 61.9%), average amino acid identity (AAI, 97.13%), TETRA (0.99%) and intergenic distance (0.04) values were below the established thresholds for differentiation. The results of the genomic analysis together with the differences in the phenotypic characteristics and the phylogenetic and chemotaxonomic analysis support the proposal of the SAICEUPSM^{T} strain as the type strain of a new species for which the name *Pseudomonas mercuritolerans* sp. is proposed. No virulence genes or transmissible resistance mechanisms have been identified, which reveals its safety for agronomic uses, under mercury stress conditions.

KEYWORDS

heavy metals, PGPB, oxidative stress protection, *Pseudomonas mercuritolerans*, phytoprotection against mercury, mercury contamination



GRAPHICAL ABSTRACT

Introduction

Up to 9,000 tons of mercury are released annually into the atmosphere, water, and soils (Zhang et al., 2021). Mercury is a global pollutant that causes damage to the environment and can affect animals and people by its transmission through the food chain. Therefore, it has the potential to become a public health and environmental threat (Ballabio et al., 2021). For this reason, there is a growing scientific, technical, and social interest in reducing mercury pollution and mitigating

its effects. Bioremediation techniques have proven to be an effective and environmentally friendly alternative (Bhatt et al., 2021) as well as useful for the recovery of high land extensions (Dary et al., 2010). *In situ* metal phytostabilization is a phytoremediation technique that uses metal-tolerant plants for the mechanical stabilization of the contaminant, preventing its transport to other environments by leaching or air transport. In addition, it reduces the accumulation of pollutants in biological systems, such as plants. Mercury is a heavy metal that has no biological role in plants or animals. For this reason, it tends to bioaccumulate, replacing other metabolically active metals in the body and triggering numerous diseases (Ballabio et al., 2021). Phytostabilization therefore sequesters contaminants in the soil environment in a more cost-effective way, especially in the case of extensive contamination. For this purpose, the use of fast-growing forage plants, such as the *Lupinus* genus, may be useful.

Phytostabilization process can be improved with the use of microorganisms with the ability to degrade contaminants and/or with the ability to promote plant growth (PGPB, plant growth promoting bacteria) under stress conditions (Kuiper et al., 2004; Azaroual et al., 2022). Traditionally, PGPB were used primarily to help plants absorb nutrients from the environment or to prevent plant diseases, in both cases by promoting their development.

Despite the natural potential of plants to remove heavy metals from the soil, phytoremediation is yet to become a commercially available technology. For this reason, it seems interesting to investigate beneficial plant-microorganism associations that improve the efficiency of the phytoremediation process. The *Pseudomonas* genus is varied and has great versatility. Many of its species can colonize different niches, due to their metabolic capacity and their ease of adaptation to different conditions (Bravakos et al., 2021). The association with plants is enhanced by the secretion of phytohormones (auxins, gibberellins, etc.), secondary metabolites (flavonoids) and enzymes (aminocyclopropane-1-carboxylate, phenylalanine ammonia-lyase) as well as siderophores, nitrogen fixation, sulfate solubilization, antibiotic production, induced systemic resistance (Sah et al., 2021; Consentino et al., 2022; Mellidou and Karamanoli, 2022), and phytopathogens control (Zhang et al., 2022). SAICEUPSM^T was isolated from the rhizosphere of a *Medicago sativa* plant native to the Almadén mining district. Specifically, it was isolated from a dump slope, where the mercury concentration was 1,071 ppm (Millán et al., 2007). Due to its ability to promote plant growth under conditions of abiotic stress, the strain was identified, and it was verified that both bioinformatics and phenotypic analysis suggest that it may be a new strain, reason why *Pseudomonas mercuritolerans* is proposed as its new taxonomic classification.

The aim of this work is the evaluation of the *in vivo* mercury phytostabilization potential under greenhouse conditions of *Lupinus albus* var., Orden Dorado plants in association with mercury resistant SAICEUPSM^T strain in soil substrates from the Almadén mining district (Ciudad Real, Spain).

Results

Isolation, identification, and phylogenetic analysis

Strain SAICEUPSM^T isolated from the rhizosphere of a *Medicago sativa* plant native to the Almadén mining district was classed as a Gram negative rod-shaped bacterium forming translucent brown colonies that intensify their hue after 72 h, ≈ 1 mm Ø, with smooth borders, and creamy texture. It grows in nutritive agar at 28°C, with aerobic metabolism, oxidase catalase positive, non-endospore-forming. Its size is 624.1–778.7 μm wide and 1.679–2.489 μm long (**Supplementary Figure 1**). These characteristics identify SAICEUPSM^T as belonging to the *Pseudomonas* genus.

The similarity analysis using the *16S rRNA* gene showed a value of 96.60% against *P. iranensis* SWRI54. All other strains analyzed showed lower similarity indices (**Table 1**).

Whole genome sequence (WGS) analysis revealed that average nucleotides identity value (ANI) value against *P. iranensis* SWRI54 was 95.34%. In the same way, the average amino acids identity (AAI) offered a value of 97.04%. For this reason, it cannot be concluded that the SAICEUPSM^T strain belongs to *P. iranensis* SWRI54. According to the

TABLE 1 Similarity of 16S rRNA with the 13 closest taxa and the SAICEUPSM^T strain.

Subject	Type strain (T)	Adhesion	Identity (%)
Pseudomonas iranensis	SWRI54	CP077092.1	96.60%
Pseudomonas allokribbensis	IzPS23	CP062252.1	90.65%
Pseudomonas hamedanensis	SWRI65	CP077091.1	90.08%
Pseudomonas kribbensis	46-2	CP029608.1	89.52%
Pseudomonas tensinigenes	ZA 5.3	CP077089.1	89.39%
Pseudomonas gozinkensis	IzPS32d	CP062253.1	88.81%
Pseudomonas glycinae	MS589	CP014205.2	87.96%
Pseudomonas zeae	OE 48.2	CP077090.1	87.82%
Pseudomonas monsensis	PGSB 8459	CP077087.1	87.27%
Pseudomonas azerbaijanoriens	SWRI123	CP077078.1	85.19%
Pseudomonas eucalypticola	NP-1	CP056030.1	84.60%
Pseudomonas mandelii	LMG26867	LT629796.1	84.74%
Pseudomonas prosekii	LMG26867	LT629762.1	84.62%

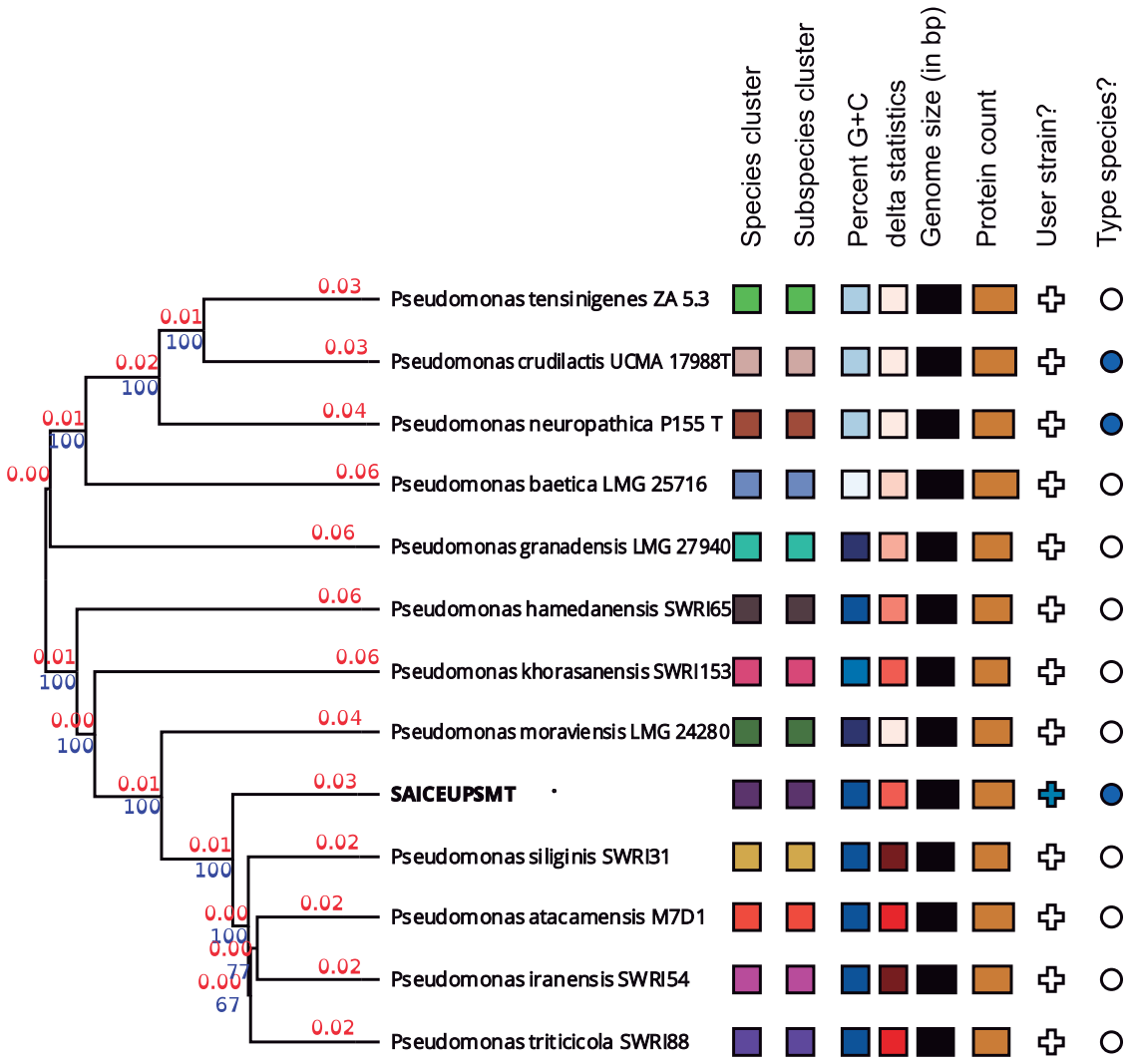


FIGURE 1
Phylogenetic tree from the complete genome of the species of the genus *Pseudomonas*. Numerical values indicate the relative distance between the analyzed species. The branch lengths are scaled in terms of Genome Blast Distance Phylogeny (GBDP) d5. The blue numbers are GBDP pseudo-bootstrap support values >60% from 100 replications, with an average branch support of 55.2%. The branch length values (in red) represent the evolutionary time between two nodes. Unit: substitutions per sequence site. The tree was rooted at the midpoint.

of the CLSI identification criteria (Clinical and Laboratory Standards Institute) strain SAICEUPSM^T could be a new species within the *Pseudomonas* genus. The phylogenetic activity of Genome Blast Distance Phylogeny (GBDP) (**Figure 1**) positioned SAICEUPSM^T within the cluster of *P. siliginis* SWRI31, *P. atacamensis* M7D1, *P. iranensis* SWRI54 and *P. triticicola* SWRI88. SAICEUPSM^T is segregated from the species in its cluster. The threshold used to discriminate nearby species is between 95–96% for ANI and 70% for DNA-DNA hybridization *in silico* (dDDH) (Richter and Rosselló-Móra, 2009). The analysis of dDDH shows that the highest value for SAICEUPSM^T is below this threshold (**Table 2**). The ANI analysis was 95.42%. These data confirm that the SAICEUPSM^T strain does not belong to any of these species (**Supplementary Table 1**: Calculated ANIb values, **Supplementary Table 2**: Calculated Tetra values, and **Supplementary Table 3**: Intergenomic distance).

Phenotypic description and plant growth promotion activities characterization

This strain is a siderophores producer and presents the ability to degrade ethylene precursor 1-aminocyclopropane-1-carboxylate deaminase (ACC) *via* enzyme ACC deaminase (ACCd). Additionally, produces the auxin class phytohormone

TABLE 2 DNA-DNA hybridization *in silico* (dDDH), confidence interval (CI), and GC percentage difference between SAICEUPSM^T and *Pseudomonas* spp. closely related.

Subject	Assembly accession	dDDH	CI	Diff GC mol %
Pseudomonas atacamensis M7D1	GCA_004801935	61.7	[58.9–64.5]	0.01
Pseudomonas iranensis SWRI54	GCA_014268585	61.5	[58.6–64.3]	0.01
Pseudomonas triticicola SWRI88	GCA_019145375	60.8	[57.9–63.6]	0.10
Pseudomonas siliginis SWRI31	GCF_019145195	59.7	[56.8–62.4]	0.09
Pseudomonas moraviensis LMG 24280	GCF_900105805	46.8	[44.2–49.4]	0.28
Pseudomonas khorasanensis SWRI153	GCA_014268505	37.6	[35.2–40.1]	0.17
Pseudomonas hamedanensis SWRI65	GCA_014268595	36.8	[34.3–39.3]	0.11
Pseudomonas granadensis LMG 27940	GCA_900105485	34.0	[31.5–36.5]	0.27
Pseudomonas tensinigenes ZA 5.3	GCA_014268445	33.6	[31.2–36.1]	0.71
Pseudomonas crudilactis UCMA 17988T	GCA_017973755	33.5	[31.1–36.0]	0.75
Pseudomonas neuropathica P155 T	GCF_015461835	33.5	[31.1–36.0]	0.66
Pseudomonas baetica LMG 25716	GCA_002813455	33.2	[30.8–35.8]	1.13

Formula d4 (a.k.a. GGDC formula 2): sum of all identities found in HSPs divided by overall HSP length.

TABLE 3 Comparison of the phenotype of SAICEUPSM^T with its closest species according to its DNA-DNA hybridization *in silico* (dDDH).

	1	2	3	4	5	6	7	8	9	10	11	12
Fluorescent pigments in King B medium (fluorescein)	+	+	−	+	−	+	+	−	+	+	+	+
Growth at 37°C	+	NA	+	−	−	−	NA	+	NA		+	+
Growth in nutritive agar + NaCl 6%	−	NA	−	/	+	+	NA	−	NA	+	+	+
Nitrate reduction	−	−	−	−	−	+	NA	−	NA	NA	+	−
Citrate utilization	+	+	+	+	NA	+	NA	+	NA	NA	NA	+
Gelatin hydrolysis	−	+	+	+	−	+	+	+	+	+	−	
D-Glucose (oxidation)	+	+	+	+	+	+	+	+	+	+	+	+
L-arabinose (oxidation)	+	+	+	−	+	+	+	+	+	+	+	−
D-galactose (oxidation)	−	+	+	+	+	+	+	+	/	/	+	−
Trehalose (oxidation)	+	+	+	−	−	NA	NA	+	NA	NA	NA	−

Taxons: 1: *Pseudomonas atacamensis* M7D1; 2: *P. moraviensis* LMG24280; 3: *P. granadensis* LMG 27940; 4: *P. baetica* LMG 25716; 5: *P. crudilactis* UCMA 17988T; 6: *P. koreensis* LMG21318; 7: *P. atagonensis* PS14; 8: *P. iridis* P42; 9: *P. allokribbensis* LMG31525T; 10: *P. gozinkensis* LMG31526; 11: *P. laurylsulfativorans* AP3_22; 12: SAICEUPSM^T; +: positive; −: negative; /: weak; NA: data is not available.

Indole-3-acetic acid (IAA, 7.72 $\mu g.ml^{-1}$). Metabolizes citrate, L-Proline aryl amidase and hydrolyzes gelatin, beta hemolytic, and urea transformer. It was negative for the motility, production of acetoin, fermentation of dulcitol, deamination of phenylalanine, production of indole, hydrogen sulfide, decarboxylation of ornithine, lysine, arginine, sucrose, xylose, maltose, mannitol, citrate, and lactose. Table 3 shows different phenotypic characteristics compared to the species with greater phylogenetic proximity.

The bacterium can grow at concentrations between 0 and 6% (p/v) of NaCl, the optimal pH is between 5.5 and 8.0 and within the temperature range of 4 to 37°C, being 28°C its optimal growth temperature. The minimum inhibitory concentrations (MIC) against different antibiotics are shown in Supplementary Table 4.

Regarding the resistance of the strain against heavy metals, quantified by calculating the minimum bactericidal concentration (MBC), SAICEUPSM^T strain was highly resistant to mercury (140 ppm), cupper (400 ppm), chrome (800 ppm), and nickel (400 ppm). In contrast, the cadmium tolerance capacity was 12.5 ppm.

Chemotaxonomic analysis

The analysis by mass spectrophotometry measured by matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) generated a list of 10 peaks based on their intensity, which allowed a peptide fingerprint and its comparison with the database (Supplementary Figure 2). The analysis of this profile showed a homologation between SAICEUPSM^T with *P. fluorescens*.

The analysis of fatty acids showed the best represented molecules: C16:0 (35.59%), Sum in Feature 3 (18.21%), C17:0 cyclo (16.73%), and Sum in Feature 8 (10.78%). The profile described by SAICEUPSM^T does not allow the identification of the strain with any nearby species (Table 4).

Genome features

The SAICEUPSM^T genome was formed from 192 contigs, with a genome length of 6,312,264 bp. The GC content was 60.07% mol (**Supplementary Figure 3A**). A total of 5,522 CDS were identified and assigned to 27 subsystems through the Rapid using Subsystem Technology (RAST) SEED viewer (**Supplementary Table 5** and **Supplementary Figure 3B**), achieving the score of 42% (2337) and through the KEGG analysis 44%. The most represented subsystems were amino acids and derivatives (481), carbohydrates (262), protein metabolism (220), cofactors, vitamins, protein, and pigments (206). The genome sequence project was deposited in NCBI (Bioproject PRJNA847155; Biosample SAMN28920853).

KofamKOALA analyses show that almost all the bacteria's major metabolic pathways were found in SAICEUPSM^T genome (**Supplementary Figure 4A** and **Supplementary Table 6**). An in-depth analysis of the mechanisms that could support the phenotypic result of resistance to mercury revealed that SAICEUPSM^T had genes from the mercury resistance operon, finding transport genes (mercuric transport protein, *merT* and mercuric transport protein, *merC*) and reduction genes (Mercuric ion reductase, *merA* and Organomercurial lyase, *merB*) (**Supplementary Figure 4B**). Likewise, the existence of genes that confer resistance to other heavy metals (cadmium, cupper, chrome, nickel), was analyzed to reinforce the phenotypic observations. The results of the whole gene collection can be consulted extensively in **Supplementary Table 7**.

The list of genes that code for mechanisms of antibiotic resistance are shown in **Supplementary Table 8**. Likewise, **Supplementary Tables 9**, **10** show the genes involved in the main virulence factors: biosynthesis of flagella proteins, adhesion motility, endotoxin, type IV pili, adherence, contraction rate, ion uptake, antiphagocytosis, and secretion systems Type I, Type II, Type III, and Type VI. These operons are incomplete, which is why the strain is not able to express them functionally. The software of the Center for Genomic Epidemiology predicted that the organism does not present a risk of pathogenesis.

In **Supplementary Table 11**, genes associated with direct and indirect mechanisms of plant growth promoters in SAICEUPSM^T are collected.

Plant growth promotion

The ANOVA analysis of the biometric variables of those plants inoculated with SAICEUPSM^T, revealed the existence of significant differences (p-value < 0.05) in the parameters total weight (Weigth_T, **Figure 2A**), root weight (Weight_R, **Figure 2B**), and number of leaves (Leaves, **Figure 2C**) compared to the controls. The SAICEUPSM^T strain significantly contributed to a higher plant weight, as well as a greater development of the root with respect to the control.

TABLE 4 Fatty acid improve titration.

	1	2	3	4	5	6	7	8
Saturated fatty acid								
$C_{10:0}$	–	–	0.1	–	–	–	–	–
$C_{12:0}$	2.40	+	1.4	1.6	2.26	1.80	1.99	4.09
$C_{14:0}$	0.53	+	0.6	–	–	+	+	–
$C_{16:0}$	35.59	24.67	33.7	32.8	36.09	31.93	32.04	–
$C_{17:0}$	0.56	–	–	–	–	–	–	–
$C_{18:0}$	0.76	–	0.4	+	–	+	+	–
Branched fatty acid								
$C_{17:0}$ cyclo	16.73	+	2.8	11.5	7.48	16.13	17.69	1.31
$C_{18:1}$ ω 7c	–	9.53	–	–	–	–	–	11.22
$C_{19:0}$ cyclo 8cω	0.89	–	–	–	–	+	+	–
Hydroxy fatty acid								
$C_{12:0}$ 2-OH	5.24	10.27	5.2	5.3	5.36	5.14	4.90	3.17
$C_{10:0}$ 3-OH	3.23	+	4.0	3.2	4.16	4.08	4.24	3.89
$C_{12:0}$ 3-OH	4.65	+	–	4.5	4.28	5.40	5.03	4.22
$C_{12:1}$ 3-OH	–	–	–	–	–	–	–	1.86
Summed features								
2	–	–	–	–	–	–	–	10.9
3	18.21		36.2	27.2	30.66	19.82	18.27	38.81
8	10.78		11.3	10.7	10.53	10.92	10.69	

Taxons: 1: *Pseudomonas atacamensis* M7D1; 2: *P. moraviensis* LMG24280; 3: *P. granadensis* LMG 27940; 4: *P. baetica* LMG 25716; 5: *P. crudilactis* UCMA 17988T; 6: *P. koreensis* LMG21318; 7: *P. atagonensis* PS14; 8: *P. iridis* P42; 9: *P. allokribbensis* LMG31525^T; 10: *P. gozinkensis* LMG31526; 11: *P. laurylsulfativorans* AP3_22; 12: SAICEU98^T; –: not detected; +: detected in small unspecified quantities.

Phytoprotection

To verify the potential oxidative stress protection effect SAICEUPSM^T strain under mercury stress conditions, the enzymatic response of *Lupinus albus* grown in soil with a high concentration of the heavy metal was evaluated. **Figure 3** shows quantification of catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GR) enzymes. Incorporating the strain into the root promotes a significant reduction in enzymatic response.

Mercury accumulation in plant

To evaluate the potential mercury phytoprotective capacity of SAICEUPSM^T, the physiological content of this heavy metal in the plant was analyzed (**Table 5**). It is observed that *Lupinus albus* tends to bioaccumulate mercury mainly at the root level. This accumulation is not statistically different in inoculated plants. The incorporation of SAICEUPSM^T,

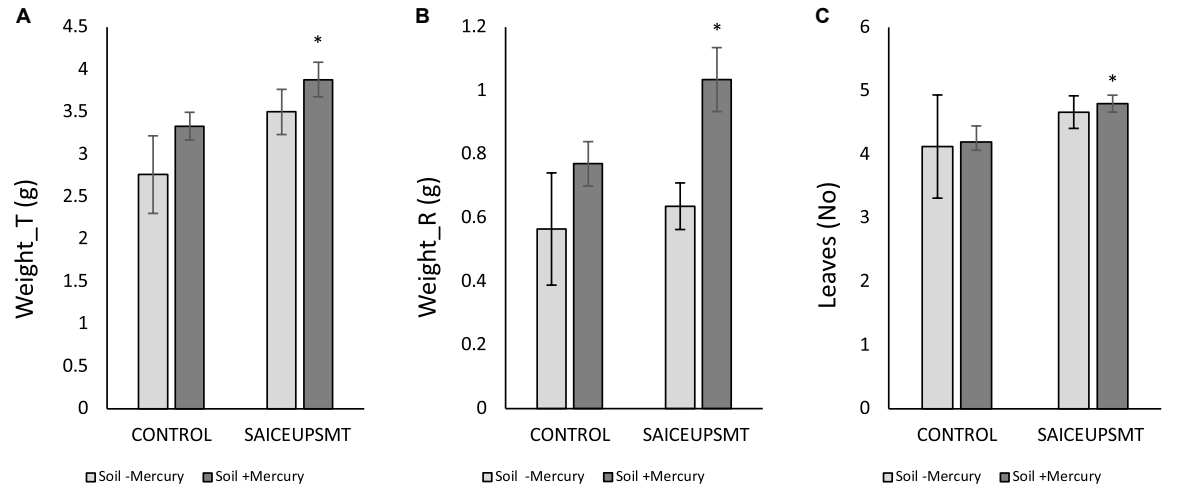


FIGURE 2
Results of the least significance difference (LSD) analysis of the biometric parameters of plants grown in soils with mercury. **(A)** Total weight, **(B)** root weight, and **(C)** total number of leaves. The bars indicate a standard error. *p-value $\leq$ 0.05. s/u, no units.

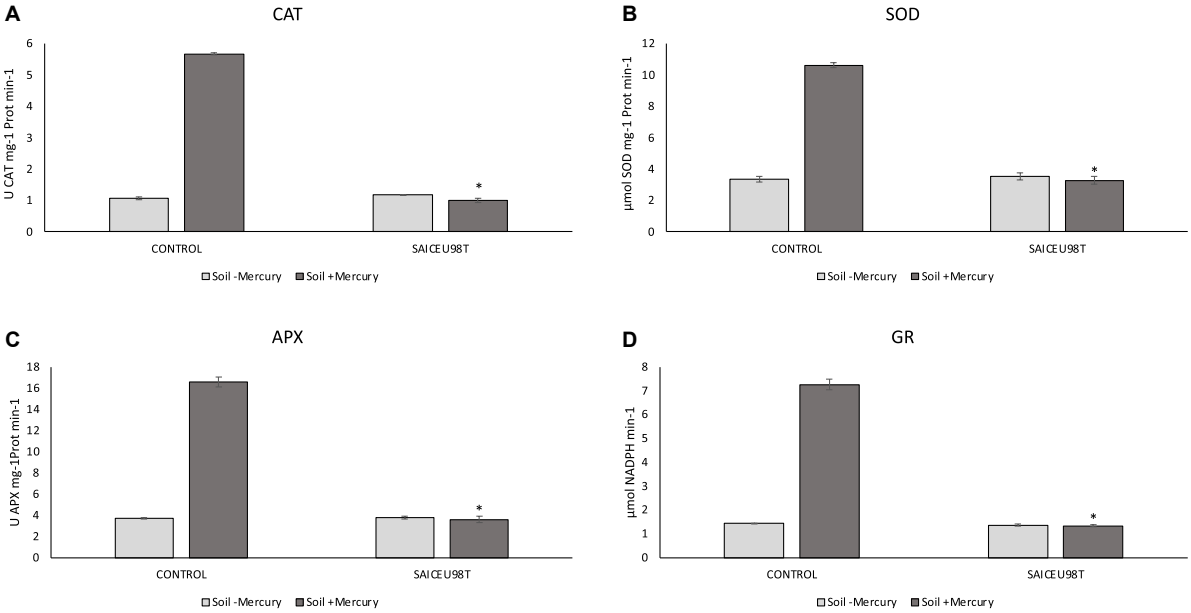


FIGURE 3
Results of Kruskal–Wallis for enzymatic content: **(A)** catalase (CAT), **(B)** superoxide dismutase (SOD), **(C)** ascorbate peroxidase (APX), and **(D)** glutathione reductase (GR). The bars indicate the standard error. *p-value $\leq$ 0.001.

promoted a significant decrease in the accumulation of mercury in the shoot of the plant, respect the control.

Discussion

Lupinus albus model has shown to be a plant with high tolerance to abiotic stress (Fumagalli et al., 2014) as well as having an interesting phytoextraction capacity (Zornoza et al., 2010; Godínez-Méndez et al., 2021; Quiñones et al., 2021). However, mercury is also known to induce physiological and metabolic alterations in plants, as the enzymatic response against oxidative stress (Zelles, 1999; Christakis et al., 2021; Çavuşoğlu et al., 2022) and decreased plant growth. Conversely, the effect of PGPB inoculation may minimize these effects on plants (Heidari and Golpayegani, 2012; Morcillo and Manzanera, 2021) in mercury-contaminated substrates (Pirzadah et al., 2018). The *Pseudomonas* genus,

precisely, has shown in previous works its important role as PGPB in stress conditions (Sandhya et al., 2010). In those plants inoculated with SAICEUPSM^T, the biometric parameters total weight, root weight and number of leaves were significantly higher in soils with high mercury concentration, compared to the same growth conditions, in the absence of PGPB inoculum. The identification of genes coding for plant growth promotion (PGP) activities explains the phenotype of the bacterium. This fact has already been described in other *Pseudomonas* species (Chellaiah, 2018; Kang et al., 2020).

From the results of this work, we confirm that, inoculation with SAICEUPSM^T strain under mercury stress produces a significant reduction in the enzymatic response to oxidative stress (CAT, APX, and SOD). SOD enzyme catalyzes the transformation of singlet oxygen to H_2O_2, with CAT and APX enzymes being responsible for eliminating H_2O_2 by transforming it into H_2O and O_2 (Gill and Tuteja, 2010). Similarly, GR is involved in reducing glutathione disulfide (GSSG) to glutathione (GSH) with NADPH expenditure. GSH plays a very important role in the redox regulation of the cell cycle and in the defense mechanisms against oxidative stress (Sánchez-Fernández et al., 1997). Plants grown in mercury contaminated soils and inoculated with SAICEUPSM^T have also significantly lower GR production. Root colonization by SAICEUPSM^T produces a phytoprotection effect against mercury stress by inducing a better antioxidant enzymatic response, compared to the non-inoculated controls.

Plants of different species, like *Lupinus albus*, can accumulate mercury in different tissues, but the absorption mechanism is still unknown (Zornoza et al., 2010; Quiñones et al., 2021; Robas et al., 2021). This fact can induce a reduction in the production of plant biomass. On the contrary, plants inoculated with SAICEUPSM^T can increase the weight and number of leaves of plants, even in substrates with high concentration of mercury. The incorporation of SAICEUPSM^T significantly reduces the absorption of mercury by the plant, while favoring its development in terms of biomass. That is why we can speak of a phytoprotective effect, which can be associated with the transformation of mercury carried out by the bacteria in the root environment, due to the expression of the genes involved in the metabolism of this heavy metal *mer* (operon). This fact is key for future agronomic applications of the strain, since the improvement in production can be significant, even under conditions of high concentration of mercury, and bioremediation processes. All of this, under safe (environmental and health) conditions since the innocuousness of the SAICEUPSM^T strain has been demonstrated as it lacks functional virulence factors and transmissible or clinically relevant resistance genes. For all the above, the SAICEUPSM^T strain is postulated as a new taxon not described to date, called *P. mercuritolerans*, with potential use in improving plant yield under abiotic stress conditions and in bioremediation processes of heavy metals, such as mercury.

TABLE 5 Mercury accumulation in plant, tested in soils with high mercury concentration.

Substrate	Inoculum	Shoot ($\mu g.g^{-1}$)	Root ($\mu g.g^{-1}$)
Soil + [mercury]	–	0.21 ± 0.02	10.06 ± 0.15
Soil + [mercury]	SAICEUPSM^T	0.13 ± 0.01*	10.04 ± 0.14

*p-value ≤ 0.001.

The growing soil heavy metal pollution in natural environments has increased scientific interest in finding alternatives for the decontamination of these ecosystems. To this end, the participation of PGPB can contribute effectively, both directly on the soil and indirectly, by contributing to the phytoprotection of plants (Singh et al., 2019). The *Pseudomonas* genus can grow and develop under unfavorable conditions, metabolize a wide variety of organic and inorganic compounds, as well as reduce and volatilize heavy metals (Beltrán-Pineda and Gómez-Rodríguez, 2016). SAICEUPSM^T strain has functional genes related to the reduction and volatilization of mercury, resistance to copper, cobalt, zinc, cadmium, as well as siderophores production capacity that help improve tolerance or transform ions of toxic metals, minimizing their toxic effects (Singh and Hiranmai, 2021), which could justify the high resistance values obtained in laboratory tests. There are few bibliographical references that review the tolerance of different species of the *Pseudomonas* genus to mercury. However, the MBC values found in this work highlight the unique resistance of SAICEUPSM^T to said heavy metal. Specifically, phenotypically, the strain ceased to be viable at a concentration of 140 ppm (516.6 μm $HgCl_2$). Sahu et al. (2016) isolated a strain that they considered to be highly tolerant to mercury isolated from an industrial effluent, capable of withstanding up to 200 μm $HgCl_2$ (i.e., almost 2.5 times less than SAICEUPSM^T). In this same sense, Zhang et al. (2012) isolated from a marine sample, a strain of *P. putida* with a tolerance to mercury like the previous authors, of 280 μm, also a much lower value than SAICEUPSM^T. This makes it an excellent candidate for the design of subsequent bioremediation processes for soils contaminated by mercury. The capacity of the strain to resist other heavy metals remains uniquely high, well above values found by other authors (Matyar et al., 2010; Singh et al., 2010) in the *Pseudomonas* genus.

Phenotypic and genotypic characterization techniques allow a multiphase approach of the strains with biotechnological interest. SAICEUPSM^T activity and the set of metabolic tests allowed it to be differentiated from nearby species, such as *P. atacamensis* M7D1. Fatty acid analysis has also been commonly used to identify species and describe new taxa (Cody et al., 2015; Heir et al., 2021). Unfortunately, this method does not always allow accurate identification. Despite the existence of undescribed profiles, the lack of homology makes us think of the possibility of the discovery of a new taxon.

Initially, the analysis of *16S rRNA*, a basic tool for classifying bacterial species, showed a similarity of 96.60% against *P. iranensis* strain SWRI54. This value is below the limits for the demarcation of species, commonly accepted (98.65%). Although the comparison of housekeeping genes is very useful for the identification of bacterial species usually isolated in the clinical field, this marker does not allow discrimination in many isolated environmental strains (Saha et al., 2019). Fortunately, the use of bioinformatics tools for the analysis of the whole genome sequence and its content has been revealed as a useful tool for discrimination and taxonomic ordering (Gutierrez-Albanchez et al., 2021).

The analysis of the total genome size of SAICEUPSM^T was comparable with that of its closest relatives, all of them belonging to the *Pseudomonas* genus. Overall genome sequence comparisons revealed an ANI value between *P. atacamensis* M7D1 and SAICEUPSM^T strain of 95.46% and a dDDH hybridization value of 61.7%. Both values are significantly below the recommended species demarcation values (8.7 and 70%, respectively) (Chun et al., 2018). This suggests that SAICEUPSM^T does not belong to any described species.

The most common mechanism of resistance to mercury in Gram negative bacteria consists of the expression and orderly participation of *merA*, *merB*, *merT*, and *merC* genes included in the *mer* operon. *merA*, has the ability to reduce Hg(II) to Hg(0), being this last one volatile. When the substrate is an organomercurial compound, such as methyl-Hg, it is *merB* that catalyzes the protonolysis cleavage of the C-Hg bond, reducing the Me-Hg to methane (CH_4), and Hg(II) (Christakis et al., 2021). The presence in SAICEUPSM^T genome of all these genes explains their mercury resilience and tolerance against. The fact that this operon does not have a repressor could also explain its high value of MCB which allows to postulate its bioremediatory application.

Antibiotics resistance genes found in SAICEUPSM^T are not transmissible and are common in *Pseudomonas* genus (Heir et al., 2021). On the other hand, genes involved in virulence mechanisms seem to lack functional capacity.

For all the above, we confirm that, inoculation with SAICEUPSM^T strain produces under mercury stress, a significant reduction in the enzymatic response to oxidative stress, significantly promotes plant fitness (increasing total weight, root weight, and number of leaves) and prevents the plant from absorbing mercury from its environment, significantly decreasing its translocation into the shoot. Therefore, SAICEUPSM^T strain is postulated as a new taxon not described to date, called *P. mercuritolerans*, with potential use in improving plant yield under abiotic stress conditions and in heavy metals bioremediation processes, such as mercury.

Description of *Pseudomonas mercuritolerans* sp. nov.

Pseudomonas mercuritolerans (adj.) refers to the strain's tolerance to mercury. It was isolated in 2014 from an autochthonous *Medicago sativa* rhizosphere grown in soils highly contaminated by mercury in the mining district of Almadén (Ciudad Real, Spain). Cells are Gram negative, aerobic, rod-shaped, ranging from 624.1−778.7 μm width to 1,679−2, 489 μm length, non-endospore forming and non-motile. The colonies grow in nutritive agar at an OGT of 28°C forming translucent, round, white-beige colonies. Growth occurs from 4 to 37°C in 24 h. It grows in a pH range of 5.5–8.0 and with a NaCl concentration of 0 to 6%. The optimal temperature, pH, and growth salinity for SAICEUPSM^T are 28°C, pH 7 and 0% salinity. It can't ferment carbohydrates, but it can use urea as a nitrogen source and citrate as the only carbon source; it is catalase and oxidase positive. It produces siderophores (pyoverdine) and can hydrolyze gelatin. The main fatty acids (>81.31% of total fatty acids) were C16:0 (35.59%), sum in feature 3 (18.21%), C17:0 cyclo (16.73%), and sum in Feature 8 (10.78%). The GC genomic DNA content of the type strain is 61.10 mole%. The whole genome sequence has been deposited in the NCBI Bioproject (PRJNA access number 847155), BioSample (SAMN 28920853).

Materials and methods

Legume plant for phytoremediation

Lupinus albus var., Orden Dorado plants were used for the *in vivo* phytoremediation experiment. Seeds were provided by the seed bank of the Center for Scientific and Technological Research of Extremadura (Spain).

Bacteria for phytoremediation

SAICEUPSM^T was selected from a larger set of isolates for its special promotion of plant growth under mercury stress conditions.

Plant growth promotion activities

To determine the *in vitro* production capacity of auxins (3-indoleacetic acid, IAA), a colorimetric technique was used with Van Urk Salkowski's reagent (Ehmann, 1977). The bacterium was grown in LB medium (Texas, USA) at 28°C for 4 days, in shaking conditions. The liquid medium was then centrifuged. In total, 1 ml of the supernatant was mixed with 2 ml of Van Urk Salkowski reagent (2% $FeCl_3$ in 35% $HClO_4$ solution) and kept in darkness. Optical density (OD) was measured at 530 nm after 30 min and 120 min. Results were quantified in ppm ($\mu g.ml^{-1}$). Glick protocol (Glick, 1995) was followed

to differentiate the degradation of ACC by the action of the enzyme ACCd of bacteria that could fix nitrogen. The culture medium contained 1.8% Bacto-Agar (Difco Laboratories, Detroit, MI, USA), low in nitrogen content, supplemented with ACC (30 mmol). The plaques were then inoculated and cultured for 3 days at 28°C, monitoring growth daily. The results were qualitatively evaluated (presence/absence of ACCd enzyme). The production of siderophores was quantified using chrome Azurol S (CAS) agar, described by Alexander and Zuberer (1991) the interpretation was based on the quantitative analysis of the production of siderophores, manifested by the appearance of a halo around the bacteria colonies after 72 h of incubation at 28°C. The ability to solubilize phosphates was tested following the protocol described by De Freitas et al. (1997). Tricalcium phosphate agar (TPM) medium was used (Nautiyal, 1999), with a final pH set to 7 with 1 mole.l^{-1} with HCl. After inoculation, the plaques were incubated at 28°C for 72 h. The inorganic phosphates solubilizer colonies showed clarification halos that were evaluated qualitatively (presence/absence). All PGPB activities were analyzed in triplicate.

Determination of mercury and other heavy metals minimum bactericidal concentration

Mercury, cadmium, copper, chrome, and nickel MBC was evaluated using Müller Hinton agar (Pronadisa®, Madrid, Spain), supplemented with different concentrations of heavy metal salts [$HgCl_2$, $CdSO_4$, $CuSO_4$, $Cr_2(SO_4)_3$, $NiSO_4$]: 800, 400, 350, 200, 175, 150, 100, 87.5, 75, 50, 43.75, and 25 μg.ml^{-1}. MBC was determined to be the lowest concentration of heavy metals salts capable of visually inhibiting >99.9% of bacterial growth after 24 h incubation. All assays were carried out in triplicate.

Substrates and setup of the experiment trays

Two types of substrates were used for the tests. The first of them was a soil with a high concentration of mercury, coming from a mining product dump (called Cerro de los Buitrones) in the Almadén mining district (38° 77′ 35″ N; 4° 85′ 07″ O, Ciudad Real, Spain). The total mercury concentration in this first substrate is 1,710 ppm (Millán et al., 2007).

The second substrate, used as a control, was a soil with a minimum natural concentration of mercury, from the area furthest, called Fuente del Jardinillo (38° 76′ 01″ N; 4° 76′ 79″ O, Ciudad Real, Spain). The use of this substrate instead of one devoid of mercury was done to guarantee the physical-chemical homogeneity of the sample. In both cases, rhizosphere-free soil samples were taken (≈50 kg per soil) from the surface down to 30–35 cm. They were transported under refrigerated conditions (4°C) to the laboratory, where they were stored (4°C) until the assay (<24 h after sampling). Both soils were sieved to guarantee the elimination of the most voluminous fractions and to homogenize the granulometry in each test and replicas.

Four sterile forest trays were used (Plásticos Solanas S.L., Zaragoza, Spain), each composed of twelve alveoli, with a capacity of 300 cm^3, with a light of 5.3 × 5.3 cm. Four pre-germinated *Lupinus albus* seeds (2.0 ± 0.5 cm emerged radicle) were sown in each alveolus.

Inoculant preparation

SAICEUPSM^T strain was incubated in nutritive agar (Pronadisa®, Madrid, Spain) supplemented with 50 ppm of $HgCl_2$ (24 h, 25°C). A bacterial suspension was prepared in 0.45% saline solution and adjusted to 0.5 McFarland (OD 10^8 cfu.ml^{-1}). It was intended to avoid an increase in salinity, which could be aggravated by the incorporation of mercury salts, and which could compromise the correct development of seedlings. Each seed was inoculated with 1 ml of suspension.

Growth under greenhouse conditions

Plants were grown for 6 weeks in a phytotron equipped with white and yellow light (photoperiod of 11 h of light, light intensity 505 μmol.m^{-2}·s^{-1}, stable room temperature 25 ± 3°C). Irrigation was performed every 48 h by capillarity with sterile tap water, with an experimental volume of 350 ml/tray.

Evaluation of the effects of mercury on plant development

Plant biometry

For biometric parameter determination, after 6-week experiment, whole plants were harvested. Roots and shoots were washed with distilled water. With the recently harvested plants, the following parameters were measured: total weight (g), shoot weight (g), root weight (g), shoot length (cm), root length (cm), total number of leaves (N_o), and total number of secondary roots (N_o).

Plant antioxidant response

Enzymes were extracted at 4°C from 1 g of fresh sample, with a mortar and using 50 mg of polyvinylpolypyrrolidone (PVPP) and 10 ml of the following medium: 50 mm of K-phosphate buffer (pH 7.8) with 0.1 mm EDTA (for SOD, CAT, and APX). The same medium, supplemented with 10 mm of β-mercaptoethanol was used for GR. Superoxide dismutase (SOD) enzyme was measured based on SOD'S ability to inhibit the reduction of tetrazoyl nitro-blue (NBT) by photochemically

generated superoxide radicals. A unit of turf is defined as the amount of enzyme needed to inhibit the rate of NBT reduction by 50% at 25°C (Burd et al., 2000). Catalase production was quantified using Aebi method (Aebi, 1984). H_2O_2 consumption was monitored for 1 min at 240 nm. This was carried out by mixing 50 mm of potassium phosphate buffer with 10 mm of H_2O_2 and 100 μl of the extract. APX content was measured in a 1 ml reaction containing 80 nm of potassium phosphate buffer, 2.5 mm of H_2O_2 and 1M sodium ascorbate. H_2O_2 was added to begin the reaction and absorbance reduction was measured for 1 min at 290 nm, to determine the oxidation ratio of ascorbate (Amako et al., 1994). Glutathione reductase (GR) enzyme was estimated spectrophotometrically, according to the Carlberg and Mannervik (1985) method at 25°C and 340 nm. To do this, the reaction mixture contained 50 mm of Tris-$MgCl_2$ buffer, 3 mm, 1 mm of GSSG, 50 μl of enzyme, and 0.3 mm NADPH, added to initiate the reaction. Enzyme concentration was calculated with the initial rate of the reaction and the molar extinction coefficient of NADPH ($\varepsilon 340 = 6.22\ mm^{-1}\ cm^{-1}$).

Mercury accumulation in plant

The root and shoot fraction of each replica were dried in dry heat furnaces at 60°C for 24 h. Each fraction was crushed and digested separately in acid medium (HNO_3/HCl 2%/0.5% weight/volume) under pressure for the determination of trace elements according to the UNE-EN 13805 standard. The product of the digestion was then analyzed by inductively coupled plasma mass spectrometry (ICP-MS). Using a calibration curve, a correlation was established between the concentration of the standard ($\mu g\ l^{-1}$) and the signal (ICP-MS) of mercury element. The element signal value in the 12 samples was interpolated on the calibration curve, resulting in the total concentration of the mercury in the sample. The mercury standard values for establishing the calibration line were as follows, expressed in $\mu g.l^{-1}$: 0.00; 0.05; 0.10; 0.50; 1.00; 5.00; 10.00. Final units' mg kg^{-1}.

$$Cf\left(\frac{\mu g}{Kg}\right) = X\left(\frac{\mu g}{L}\right) \cdot D \cdot \frac{V\,(mL)}{W\,(g)} \cdot 10^{-3}$$

Cf (mg kg^{-1}) is the metal content of the sample, X ($\mu g\ l^{-1}$) corresponds to the interpolated experimental value or the extrapolated experimental value of the standard addition; D is the dilution factor; V (mL) is the volume of the flask, and W(g) to the weight of the sample.

SAICEUPSM^T strain identification

Transmission electron microscopy (TEM)

To determine the size and shape of the analyzed strain, the Prism E scanning electron microscope (SEM) (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used. The culture was observed in suspension. A drop of Formvar was used in transmission electron microscopy (TEM) Cu grids of mesh 200 as a sample support in the grid for TEM microscopy. The measurement conditions were working distance of 10 mm; 24 pA electron current; electron acceleration of 30 kV and size of 2 points, pressure of 375 Pa, at 4°C and a humidity of 50%. The electron microscopy images were obtained by the research support service (SAI) of "X-ray diffraction and scanning electron microscopy" (SAI-DRX-MEB) of the San Pablo CEU University (Madrid, Spain).

Biochemical tests

Oxi/Ferm Pluri Test® (Liofilchem, Italy) was used. Next, the automatic characterization was carried out with the VITEK® 2 equipment and with the VITEK® 2 GN identification cards (bioMérieux, Marcy-l'Étoile, France). The motility of the bacterium was tested in Motility Test Agar, (Liofilchem, Italy). Antimicrobial sensitivity was determined using *E*-test in Müller Hinton agar (Pronadisa®, Madrid, Spain) using the following antibiotics: piperacillin and piperacillin with tazobactam, cefepime (bioMérieux, Marcy-l'Étoile, France); ceftazidime, imipenem, imipenem with EDTA, amikacin, gentamicin, and ciprofloxacin (Liofilchem, Italy).

Fatty acids

The analysis of cellular fatty acids was carried out in the Spanish Collection of Type Crops (CECT) at the University of Valencia, Spain. Cells were grown in M2 medium for 48 h, 30°C; extractions and determinations were carried out according to the standard protocol of the MIDI Microbial Identification System (Sasser, 1990) using a chromatograph Agilent 6850 (Agilent Technologies) following the method TSBA6 (MIDI, 2008, version 6.1. Newark, DE: MIDI Inc.).

Phylogenetic analysis

Matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) was used. It was carried out in the Vitek MS IND system (BioMérieux, Marcy-l'Étoile, France) at the Carlos III Health Institute (Majadahonda, Madrid). Slides were inoculated with a sterile handle. A total of 1 μl of the matrix solution (VITEK MS-CHCA: mixture of 3.10 g of 2.5-dihydroxy 36 benzoic acid dissolved in 100 ml of water-ethanol-acetonitrile in 1/1/1 ratio) was added to each well and allowed to dry at room temperature. the temperature. Mass spectra were generated with the Axima Assurance system (Shimadzu Corporation, Kyoto, Japan), using the Shimadzu Launchpad software program and the SARAMIS MS-ID v1 database application (AnagnosTee GmbH) for automatic measurement and identification. All strains were analyzed in duplicate. No pre-treatment was used before inoculation on the slide. High confidence identification was considered when the assessment was equal to or greater than 97%.

SAICEUPSM^T genomic DNA was extracted from fresh cells. Amplification of the *16S rRNA* gene was done and BLAST algorithm was used to search for similar sequences. An *in silico* genome analysis was carried out among the most closely related species, for this purpose the Type (Strain) Genome Server (TYGS) service was used using blast+ software. The resulting intergenic distances were used to infer a balanced evolution with branch support through FASTME 2.1.6.1. Compatibility was inferred from 100 pseudo-bootstrap replicas. Trees were rooted in the midpoint and visualized with PhyD3. For the calculation of ANI and AAI the tools available in JSpeciesWS and the ANI calculator[1] were used.

Genome sequencing and bioinformatics analysis

For the extraction of genomic DNA, the QIAamp DNA Kit (QIAGEN®, Hilden, Germany) was used. SAICEUPSM^T genome was obtained by whole genome sequencing using an Illumina Miseq platform. A total of 200 ng of genomic DNA were used, measured by fluorimetry (Quant-iT Picogreen, Thermo Fisher). To make libraries, the NEB Next ultra II FS DNA preparation kit was used, according to the manufacturer's protocol (New England Biolabs). The initial fragmentation time was 7.5 min, and the final PCR amplification was done with six cycles. The resulting DNA fragments were evaluated and quantified by bioanalyzer using a 7500 DNA (Agilent) chip. The libraries were quantified by qPCR using the master mix "Kapa-SYBR FAST qPCR kit for LightCycler480." Libraries were sequenced in Illumina's Miseq equipment following manufacturer's instructions, in a pair end 2 × 300 type race using "Miseq reagent kit v3 600 cycles."

The quality of the sequenced reads was processed using FastQC v.0.11.3 (Babraham Bioinformatics) (Andrews, 2010). They were filtered for low quality reads using Prinseq (Schmieder and Edwards, 2011) and adapter regions using CUTADAPT (Martin, 2011). To eliminate duplicate reads or those that were only present in forward or reverse, FASTQCollapser and FASTQIntersect were used. The *de novo* assembly of the genome was performed with SPAdes v.3.13 (Center for Algorithmic Biotechnology) (Bankevich et al., 2012), the metrics of the assemblies were obtained through SeqEditor (Hafez et al., 2021) and annotated using the Prokka software, version 1.13[2] (Seemann, 2014) and Rapid using Subsystem Technology (RAST) version 2.0[3] with predetermined parameters (Aziz et al., 2012). The genomes of the related strains were obtained from the Genbank database.

Additional bioinformatic analyses were performed. tRNAscan-SE v.2.0[4] was used to predict tRNAs. Several clinically important antimicrobial resistance genes and virulence determinants were searched through functional annotation data generated from Rast, Prokka, and ResFinder 4.1 annotation lines. Several mercury resistance and plant growth promotion genes were searched through functional annotation data generated from Rast and Prokka annotation lines. KEGG software was used to examine the metabolic pathways of the strain, and PathogenFinder v.1.1., Rast and Prokka software were used to estimate pathogenicity.

Statistical analysis

The Kolmogorov–Smirnov test was performed to check the normality of all variables. After that, for the analysis of the biometric data, an ANOVA test was carried out to analyze the plant response in the presence of SAICEUPSM^T in substrates under high and low mercury concentration. For those parameters that showed statistical significance (p-value ≤ 0.05), the multiple comparison test least significant difference (LSD) was performed, to identify if SAICEUPSM^T produced any significant variation in plant biometry, compared to controls. For the analysis of the phytoprotective capacity, a Kruskal–Wallis analysis was performed. For the evaluation of mercury accumulation in plants, the normality of the sample data was verified using the Shapiro–Wilk test. An ANOVA analysis was then performed to determine the existence of significant differences (p-value ≤ 0.05) between treatments. All statistical differences refer to the comparison of the variables when the plant has been inoculated, compared to their respective non-inoculated controls. SPSS v.27.0 software was used (Version 27.0 IBM Corp., Armonk, NY, USA).

Data availability statement

The data presented in this study are deposited in the Genbank repository, accession number: JAMSHA000000000.

Author contributions

PJ, VF, and MR: conceptualization and methodology. VF: software. PJ and MR: validation and supervision. DG, VF, and LG: formal analysis. DG, VF, LG, and MR: investigation. VF and LG: resources. PJ, VF, and LG: data curation. LG and MR: writing—original draft preparation and visualization.

1 http://enve-omics.ce.gatech.edu/ani/, accessed 5 July 2022.

2 https://github.com/tseemann/prokka

3 https://rast.nmpdr.org/

4 http://lowelab.ucsc.edu/tRNAscan-SE/

PJ, AP, and MR: writing—review and editing. AP and PJ: project administration and funding acquisition. All authors read and agreed to the published version of the manuscript.

Funding

This research was funded by Fundación Universitaria San Pablo CEU (Microbiology SAI) and Banco Santander, grant number: FUSP-BS-PPC01/2014.

Acknowledgments

We are grateful to the Seed Bank of the Center for Scientific and Technological Research of Extremadura, for its permanent availability in the supply of seeds for trials. Special thanks to the Carlos III Health Institute, Laboratory of Bacterial Taxonomy (Majadahonda, Madrid, Spain), for helping us in sequencing the complete genome of the strain. Especially very thank to Sylvia Valdezate Ramos, María José Medina Pascual, and Gema Carrasco Diaz.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1032901/full#supplementary-material

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III. Conclusiones

La estrategia planteada en la presente tesis doctoral ha sido profundizar en el conocimiento de las comunidades microbianas edáficas de suelos contaminados por Hg; la selección, caracterización y ensayo de PGPR capaces de inducir fitoprotección, así como la evaluación del impacto biológico en el entorno planta-microorganismo por la adición de dichas bacterias, al objeto de ensayar procesos ulteriores de fitorremediación y/o fitorrizoremediación. El área escogida para el trabajo (Distrito Minero de Almadén) ofrece un ambiente sujeto a una presión selectiva intensa y continuada durante un periodo de siglos, tanto para para plantas como para microorganismos edáficos. Así, el trabajo que ahora se presenta posee tres grandes bloques de investigación que se desarrollan de forma transversal en los diferentes apartados de la tesis:

1. Aislamiento, identificación, caracterización PGPR con potencial uso biotecnológico, mediante la aplicación adaptada del IIBMR, de una parte, así como la descripción y clasificación de nuevos taxones bacterianos, de otro.

2. Estudio de las modificaciones en la composición, funcionalidad y fenotipo de las comunidades bacterianas del suelo de Almadén como resultado de la inoculación de PGPRs individualmente y consorciadas, a través de dos vías: i) análisis metagenómico descriptivo de los suelos mediante la técnica de *Shotgun* y ii) el ensayo y propuesta de la técnica del Cenoantibiograma como nuevo indicador para el seguimiento de la evolución del tratamiento biológico del suelo

3. Estudio de fitoproteccion y fitorrizorremediación frente al Hg mediante el empleo de PGPR.

Para el desarrollo de estos tres grandes bloques de investigación se plantearon cinco objetivos. Los experimentos desarrollados conducen a las siguientes conclusiones:

1. En el **estudio metagenómico comparativo** de las **comunidades bacterianas rizosféricas** mercuroresistentes vinculadas a plantas que crecen de forma natural en Almadén **frente a comunidades microbianas de suelo libre**, **se constata** que determinados taxones bacterianos se seleccionan conforme a los diferentes ambientes. Así, las **Beta y Gammaproteobacteria se encuentran íntimamente ligadas a suelo rizosférico**, mientras que **Acidobacteria y Cyanobacteria tienen una mayor representación en suelo libre**. **El análisis del perfil funcional confirma la co-selección** de los genes codificantes para la resistencia a antibióticos junto con aquellos que permiten la resistencia a otros compuestos tóxicos tales como el Hg. Por tanto, **se evidencia que la contaminación por Hg** ejerce una presión selectiva que favorece el papel **como reservorio de mecanismos de resistencias a antibióticos** en **dichos suelos.**

2. **El IIBRM,** cuando se evalúa en medios adicionados con Hg**, ha demostrado ser una herramienta útil para evaluar de forma integrada las capacidades PGP**. Se propone como criterio de selección de cepas para ulteriores usos biotecnológicos aquellas bacterias que, valoradas sus capacidades en medios adicionados con Hg, presenten **una CMB-Hg > 100 µg/mL así como un IIBMR > 6.5**. Con base en dicho criterio, se han podido seleccionar **cuatro cepas bacterianas con un alto potencial biorremediador**. La secuenciación de la región V1-V9 del gen codificante para el *16S rRNA* asigna su identificación como: *Bacillus toyonensis*, *Pseudomonas moraviensis* (posteriormente clasificada como *Pseudomonas mercuritolerans sp. nov.)*, *Pseudomonas baetica* y *Brevibacterium frigoritolerans.*

3. El **comportamiento biológico** de las plantas de *Lupinus albus* var. Orden Dorado (biometría, bioacumulación de Hg y actividad enzimática antioxidante) tras su inoculación con PGPR evidencia que:

    a. La cepa de ***Pseudomonas baetica,*** así como sus respectivos consorcios tanto con *Pseudomonas mercuritolerans sp. nov.* como con *Brevibacterium frigoritolerans* son **capaces de promover el crecimiento vegetal en condiciones edáficas adversas** por contaminación con mercurio.

    b. Las cepas de ***Pseudomonas baetica y Pseudomonas mercuritolerans***, tanto de manera individual como consorciada son capaces de i) **reducir significativamente el estrés oxidativo** de la planta para las actividades catalizadas por las enzimas catalasa (CAT), superóxido dismutasa (SOD), ascorbato peroxidasa (APX) y glutatión reductasa (GR) y ii) ejercer un **efecto fitoprotector, disminuyendo la acumulación sistémica del Hg en la planta**.

4. La secuenciación masiva del genoma de las dos cepas de *Pseudomonas* ensayadas revela que **ninguna de estas bacterias posee genes de virulencia** que puedan expresarse, hecho que evidencia su inocuidad. Ambas poseen un **elevado potencial genético de promoción del crecimiento vegetal**. Así mismo, el genoma de **la cepa de *Pseudomonas moraviensis*** posee un **bajo índice de homología con los genomas de referencia**. Análisis más exhaustivos de dDDH y ANI nos permite describirla y clasificarla como la cepa tipo de la especie ***Pseudomonas mercuritolerans* sp nov**. Así mismo, podemos concluir que **la secuenciación de gen codificante para el *16S rRNA* no resulta idóneo por sí mismo para la identificación de cepas ambientales sometidas de manera crónica a contaminación por Hg.**

5. El empleo de la **técnica del cenoantibiograma** demuestra que, la adición de la cepa de *Brevibacterium frigoritolerans* a la rizosfera de *Lupinus albus* var. Orden Dorado, así como los consorcios en los que participa, **son capaces de modificar el perfil de**

resistencias a antibióticos de la comunidad microbiana edáfica, reduciendo de forma significativa las CMI frente a los antibióticos de uso más extendido en clínica. La técnica de cenoantibiograma **ha demostrado ser una herramienta útil para evaluar el impacto de la adición de una cepa bacteriana en la expresión fenotípica de la resistencia a antibióticos medidos en términos de CMI.**