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ANÁLISIS DE LA DIVERSIDAD GENÓMICA DE AISLADOS DE *ACINETOBACTER HAEMOLYTICUS*

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Abstract

Acinetobacter haemolyticus is a Gram-negative bacterium associated to nosocomial opportunistic infections. Even though most of the isolated strains are antibiotic-sensitive, there have been an increase in the reported cases, worldwide, where *A. haemolyticus* carries clinically relevant antibiotic resistance determinants, such as the NDM-1 metallo-beta-lactamase, which confers resistance to carbapenems, one of the antibiotics used to treat *Acinetobacter* infections. In this work, I analyzed the complete genomes of 32 Mexican *A. haemolyticus* strains (coming from different hospitals) and 12 strains isolated in other parts of the world (available from the RefSeq database) to analyze the genomic diversity in this genome collection, with an emphasis on the evolutionary forces that shaped its chromosome's architecture, such as horizontal gene transfer and gene gain and loss. This study shows that *A. haemolyticus* have an open pangenome and that there are different lineages circulating in Mexican and Chinese hospitals. Moreover, the *A. haemolyticus* chromosome is formed by 12 syntenic blocks, which contain most of the core genes, separated by hypervariable regions, which differ in size and genome content. Finally, most of the genes that code for known virulence factors lie in the chromosome, but antibiotic resistance determinants are found in the chromosome and in plasmids; thus, monitoring of this species in the hospitals is needed.

Resumen

Acinetobacter haemolyticus es una bacteria Gram negativa que se asocia a infecciones oportunistas en los hospitales. Si bien la mayoría de las cepas aisladas son sensibles a los antibióticos comúnmente utilizados, ha habido un aumento en los casos reportados a nivel mundial en donde *A. haemolyticus* porta determinantes de resistencia clínicamente relevantes, tales como la metalo-beta-lactamasa NDM-1, que confiere resistencia a los carbapenémicos, uno de los antibióticos utilizados para el tratamiento de infecciones por bacterias del género *Acinetobacter*. En el presente trabajo analicé los genomas completos de 32 cepas mexicanas de *A. haemolyticus* (provenientes de distintos hospitales) y 12 cepas aisladas en otras partes del mundo (tomadas de la base de datos de RefSeq) para estudiar la diversidad genómica presente en ese conjunto de cepas, con especial énfasis en las fuerzas evolutivas que han modelado la arquitectura de su cromosoma, tales como la transferencia horizontal y la pérdida y la ganancia de genes. Este análisis muestra que *A. haemolyticus* tiene un pangenoma abierto y que hay distintos linajes circulando en los hospitales mexicanos y chinos, en lugar de una sola clona. Además, el cromosoma de *A. haemolyticus* se compone por 12 bloques sinténicos, los cuales contienen la mayor parte de los genes core, separados por regiones altamente variables en tamaño y en contenido génico, las cuales tienen señales de transferencia horizontal. Finalmente, la mayoría de los genes que codifican para factores de virulencia conocidos se encuentran en el cromosoma; pero los genes de resistencia a antibióticos se encuentran tanto en el cromosoma como en los plásmidos, por lo que se necesita monitorear a esta especie.

Introducción

Las bacterias se encuentran en gran abundancia en el ambiente, y pueden ser parte importante de nuestras vidas. Por ejemplo, algunas bacterias de nuestra microbiota intestinal producen hasta una cuarta parte de los requerimientos diarios de las vitaminas K y del complejo B (Rowland et al., 2018). Sin embargo, también existen bacterias patógenas que causan daño al hospedero. Entre los elementos que determinan si una bacteria será patógena, se encuentran el estado del hospedero y la presencia y expresión de factores de virulencia en la bacteria (Casadevall and Pirofski, 2003, 2019).

La genómica comparativa permite comparar el genoma de organismos de interés a nivel de un conjunto de genes, de operones, de islas genómicas, o de plásmidos. Actualmente es posible ensamblar y anotar múltiples genomas, y hacer estudios a distintos niveles taxonómicos porque el costo de secuenciar el genoma completo de varias bacterias ya es más accesible para grupos de investigación, en varias partes del mundo (Land et al., 2015; Wick and Holt, 2019). Asimismo, la tecnología para estudiarlos también se ha hecho accesible gracias a los avances tecnológicos a nivel de cómputo y de almacenamiento de datos (Dominguez Del Angel et al., 2018; Wick and Holt, 2019).

Cuando se analizaron los primeros genomas de varias cepas de una misma especie, y después de múltiples especies del mismo género, se resaltó que no todas las cepas de una misma especie contienen los mismos genes. Entonces, se acuñó el término de "pangenoma" para describir al conjunto completo de genes que hay en el grupo de comparación (ya sea a nivel de grupo de aislados, a nivel de especie, de género, o de mayor orden taxonómico), que si lo viéramos como conjuntos, se trataría de la unión, y de "genoma *core*" para englobar a todos los genes compartidos en un mismo grupo de estudio, que sería la intersección (Tettelin et al., 2005; Vernikos et al., 2015). Así que, las diferencias en contenido génico, ya sea a nivel de cromosomas o de plásmidos u otros elementos móviles, pudieran contribuir a diferencias fenotípicas, tales como la patogenicidad o el grado de virulencia de una bacteria y la agresividad de la infección que causa (Rosenstein and Götz, 2012). De hecho, los genes de resistencia a los antibióticos, a los metales, y a los antisépticos les pueden dar ventajas a las bacterias patógenas en el ambiente (como sitios contaminados) así como en los hospitales.

A partir de los cambios observados a nivel molecular, ya sea a nivel de la secuencia de ADN o de proteínas, podemos trazar las relaciones ancestro-descendiente entre organismos y lo representamos con un árbol filogenético. Gráficamente, los nodos terminales u hojas sería lo que estamos estudiando, y pueden representar organismos (*taxa*), proteínas, o genes; y los nodos más internos representarían al ancestro común; la longitud de las ramas puede indicar qué tan diferentes son los nodos terminales entre sí (Podsiadło and Polz-Dacewicz, 2013). Para analizar conjuntos grandes de datos se usan métodos heurísticos, los cuales exploran algunas soluciones y regresan un resultado que podría o no ser el mejor. Se usa esta aproximación porque no es viable hacer una búsqueda exhaustiva con conjuntos de datos masivos, pues la cantidad posible de árboles aumenta rápidamente con el número de *taxa* a analizar (Yang and Rannala, 2012). Por ejemplo, para tres *taxa* hay 3 árboles posibles, mientras que para 4, 5 y 6 ya son 15, 105 y 945 respectivamente (Drummond and Bouckaert, 2015). Además, un solo marcador genético no nos da suficiente información para distinguir entre cepas muy parecidas; más *loci* significa mayor información. Entonces, podemos inferir un árbol filogenético más confiable si juntamos los alineamientos de varios *loci* (Yang and Rannala, 2012; Amit Roy, 2014).

En estudios epidemiológicos podemos apoyarnos de la genómica comparativa y de la filogenética para trazar el origen de un brote, que es el aumento del número casos de una enfermedad por encima de lo esperado en un período de tiempo determinado en un lugar -hospital, estado, o país- (World Health Organization), pues esperaríamos que los genomas fueran idénticos o muy similares entre las cepas aisladas del origen y las que infectaron a los pacientes. Por ejemplo, en distintos estudios de brotes de *Salmonella* asociadas a alimentos, se pudieron trazar el origen u orígenes, así como distinguir de manera más precisa los casos asociados a los brotes de aquellos no relacionados a los mismos (Besser, 2018; Pearce et al., 2018). Las diferencias se pueden evaluar a nivel de cambios de una sola o pocas bases en la secuencia de ADN, rearreglos genómicos, y de contenido génico.

Durante el doctorado participé estrechamente en el estudio de varias bacterias patógenas; pero el eje central de mi tesis se centró en la identificación de los genes que le pudieran brindar a *Acinetobacter haemolyticus*, un patógeno emergente y poco estudiado, alguna ventaja en el ambiente hospitalario, como pudieran ser los genes de resistencia a

antibióticos, diversos factores de virulencia y elementos adquiridos por transferencia genética horizontal. También puse especial empeño en estudiar la arquitectura del cromosoma de estas bacterias, sobre todo en la organización y contenido génico de los bloques sinténicos que lo conforman. Durante mi doctorado colaboré en varios proyectos donde analizamos diversos aspectos de la arquitectura genómica de *Acinetobacter baumannii*, un patógeno especialmente problemático en los hospitales. Los artículos que salieron como producto de estas colaboraciones los añado como apéndices de la presente tesis (Apéndices 1 a 8).

Las infecciones oportunistas

Las personas con sistemas inmunes comprometidos son más susceptibles a padecer infecciones oportunistas, las cuales no ocurren en los hospederos sanos (Rali et al., 2016). Un paciente puede estar inmunocomprometido debido a la desnutrición, pero también por el uso de inmunosupresores que se requieren en el tratamiento de enfermedades autoinmunes o para evitar el rechazo de los trasplantes (George et al., 2014; Rali et al., 2016; Padayachy and Graham Fieggen, 2018). Otras causas de la inmunosupresión incluyen las quimioterapias que se utilizan en el tratamiento del cáncer, la manifestación del SIDA, (Síndrome de Inmunodeficiencia Adquirida), debido al virus del VIH (Virus de la Inmunodeficiencia Humana) y las inmunodeficiencias congénitas (George et al., 2014; Teillant et al., 2015; Rali et al., 2016; Padayachy and Graham Fieggen, 2018). Asimismo, algunos pacientes, como aquellos internados en las unidades de cuidados intensivos, son especialmente susceptibles a las infecciones oportunistas porque sufrieron heridas extensas y graves (como quemaduras o fracturas expuestas), o han sido sometidos a procedimientos invasivos como pudiera ser la colocación de catéteres, la ventilación mecánica, la cirugía abdominal o colo-rectal (Teillant et al., 2015).

El género *Acinetobacter*

El género *Acinetobacter* está conformado por bacterias Gram negativas, catalasa-positivas, oxidasa-negativas, del orden de las Gammaproteobacterias. Se han descrito poco más de 63 especies aceptadas dentro del género *Acinetobacter* (https://apps.szu.cz/anemec/Classification.pdf), y ese número crece conforme se van aislando cepas de distintos ambientes y en la medida de que los aislados se caracterizan con más profundidad, sobre todo aquellos que inicialmente sólo se identificaron a nivel de género. Entre los nichos que los miembros del género *Acinetobacter* ocupan, podemos encontrar a los alimentos (Rafei et al., 2015), los ríos (Icgen and Yilmaz, 2014; Maravić et al., 2016), el suelo (Vangnai et al., 2007; Mujumdar et al., 2014; Rafei et al., 2015), los animales (Guardabassi et al., 1999; Müller et al., 2014; Rafei et al., 2015) y en el drenaje de algunos hospitales (Hu et al., 2017, 2018, 2019; Qin et al., 2018). Hay que recalcar que

algunas especies son patógenas oportunistas en humanos (Wong et al., 2016; Shin and Park, 2017).

El género *Acinetobacter* se divide en tres clados principales (Touchon et al., 2014; Garcia-Garcera et al., 2017). El clado de *A. baumannii* contiene especies que son principalmente patógenos oportunistas, pero algunas se encuentran en el ambiente. El clado hemolítico tiene especies que principalmente son ambientales, pero que en ocasiones pueden ser patógenas, y el otro clado tiene especies que principalmente son ambientales. Hay además otras pocas especies que no pertenecen a ninguno de esos clados, de las cuales sólo dos se asocian a infecciones en humanos (*A. soli* y *A. ursingii*).

Hasta el 13 de mayo de 2020, había 6290 genomas secuenciados del género *Acinetobacter* depositados en la base de datos de Genbank, donde la mayoría son de *A. baumannii* (4754 genomas; es decir, el 75.6%), mientras que para especies poco estudiadas sólo hay de uno a una decena de genomas, y para el resto de las especies fuera del complejo *Acinetobacter calcoaceticus-baumannii*, no pasan de 100 genomas, tal como se observa en la Figura 1.

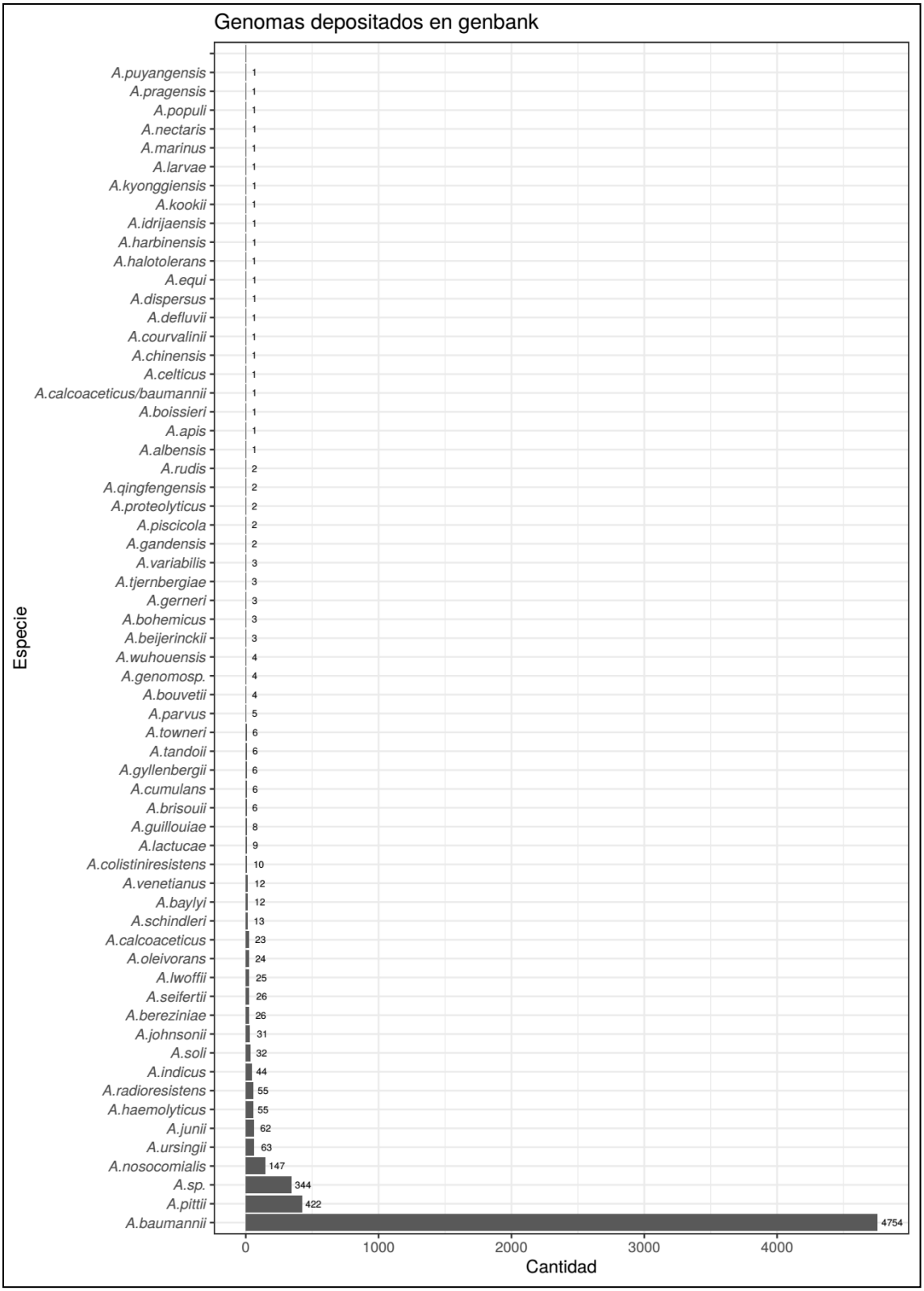

Figura 1. Cantidad de genomas del género *Acinetobacter* depositados en Genbank.

Identificación de especies dentro del género *Acinetobacter*

Actualmente, para identificar especies dentro del género *Acinetobacter* se utilizan pruebas bioquímicas, o el perfil de proteínas obtenidas por espectrometría de masas, o datos de secuenciación de uno, pocos genes, o de genomas completos (Rafei et al., 2014, 2019; Vijayakumar et al., 2019). Estas aproximaciones se pueden utilizar en combinación, o de manera gradual (de menor a mayor detalle), para dar una asignación más precisa de a qué especie pertenecen los aislados bajo estudio.

Con las pruebas bioquímicas se evalúa el aprovechamiento de fuentes de carbono y otros nutrientes del aislado en cuestión. Sin embargo, hay aislados que pueden tener perfiles parecidos a los de una especie a la que no se parecen tanto a nivel de secuencia. Esto pudiera deberse a la variación fenotípica dentro de la especie (Sutcliffe, 2015; Vijayakumar et al., 2019), ya sea por mecanismos regulatorios, la inactivación de enzimas, o la adquisición de genes por transferencia horizontal. Muchos laboratorios de microbiología clínica en los hospitales utilizan estas pruebas porque, si bien no siempre aciertan a nivel de especie, pueden guiar el tratamiento a partir del antibiograma y con la información a nivel de género que se tiene. Asimismo, existen *kits* comerciales para automatizar o estandarizar estas pruebas, lo que hace que puedan utilizarse de manera rutinaria para grandes cantidades de muestras diarias.

Por medio de la espectrometría de masas se evalúa el perfil de proteínas, por ejemplo, las ribosomales que son las más abundantes en la célula, y se contrasta con la información depositada en bases de datos. La calidad de la asignación depende de qué tan poblada y actualizada se encuentre la base de datos, así como la versión del equipo, y las condiciones experimentales (Šedo et al., 2018). Cabe destacar que estas pruebas sólo se pueden hacer en los hospitales grandes o en los centros de referencia, pues el equipo de espectrometría de masas es bastante costoso.

Para identificar especies dentro del género *Acinetobacter* con datos de secuenciación de DNA, podemos usar uno o pocos genes monocopia, o aprovechar la información del genoma completo. Uno de los marcadores más utilizados para identificar especies dentro del género *Acinetobacter* es un fragmento del gen que codifica para la subunidad beta de la

RNA polimerasa, *rpoB*, y en general, da resultados confiables (Gundi et al., 2009; Vijayakumar et al., 2019).

Por otro lado, para estudiar específicamente a *A. baumannii* y a otras especies muy cercanas a ésta, existe la tipificación de secuencias por multi *locus*, del inglés *Multi Locus Sequence Typing* (MLST), en el cual se analizan 7 fragmentos de genes *core*. Existen dos esquemas, Oxford y Pasteur, que comparten un par de marcadores. Ambos esquemas, así como estudios previos con otras aproximaciones y estudios posteriores con análisis de genomas completos, coinciden en que en *A. baumannii* hay cepas muy relacionadas genéticamente (clonales) y que se encuentran diseminadas a nivel internacional; entre ellas, las más importantes son GC1 y GC2, del inglés, *Global Clone* (Bartual et al., 2005; Diancourt et al., 2010). Los dos esquemas de MLST, pero en especial el esquema Oxford, pueden dar relaciones erróneas si algunos de los *loci* utilizados sufrieron recombinación (Hamidian et al., 2017; Castillo-Ramírez and Graña-Miraglia, 2019; Gaiarsa et al., 2019). Además, el esquema Pasteur a veces engloba en un solo grupo a cepas que en realidad son muy diferentes, pues los marcadores que utiliza tienen muy poca variación genética (Castillo-Ramírez and Graña-Miraglia, 2019; Gaiarsa et al., 2019).

Los estudios iniciales del género *Acinetobacter* a nivel de genomas completos comparaban a *A. baylyi* ADP1, que no es patógena, con las cepas de *A. baumannii* sensibles y resistentes a diversos antibióticos, en busca de diferencias a nivel de secuencia del ADN que reflejaran las diferencias en los fenotipos. En estos estudios se encontró que *A. baumannii* tenía más factores de virulencia (Smith et al., 2007), y que la estrategia de adquisición de hierro era distinta entre estas dos especies (Vallenet et al., 2008). Además, encontraron que en las bacterias resistentes (que también era epidémicas) había islas genómicas en las que se acumulaban genes de resistencia a antibióticos, lo cual les daba ventaja en los hospitales (Fournier et al., 2006; Iacono et al., 2008; Liu et al., 2014).

Se sabe que *A. baumannii* tiene gran variación en el contenido génico, inclusive entre cepas muy relacionadas filogenéticamente como sucede en los brotes de los hospitales; entre los genes que más varían se encuentran aquellos asociados con resistencia a antibióticos, enzimas relacionadas con la síntesis de polisacáridos de la superficie celular o de la

cápsula, y elementos genéticos móviles como secuencias de inserción (IS), bacteriófagos, y plásmidos (Wright et al., 2014; Graña-Miraglia et al., 2017; Mancilla-Rojano et al., 2019).

Islas genómicas

Algunos factores de virulencia y genes de resistencia a antibióticos y a otras moléculas tóxicas pueden encontrarse en islas genómicas, las cuales son regiones que albergan uno o varios genes relacionados con el fenotipo de estudio y tienen huellas de un posible origen por transferencia genética horizontal, pues tienen un contenido atípico de GC y de uso de codones, y están presentes tan sólo en un subconjunto de las cepas, usualmente sólo las patógenas o en las resistentes a antibióticos, según sea el caso (Hacker and Kaper, 2000; Dobrindt et al., 2004; Partridge et al., 2018).

Las islas genómicas más estudiadas en el género *Acinetobacter* son las islas de resistencia, inicialmente caracterizadas en *A. baumannii*, por lo que se nombraron con el prefijo "AbaR". Las islas tipo AbaR se componen de uno o varios genes de resistencia a antibióticos y están flanqueadas por regiones conservadas, que incluyen repetidos invertidos y subunidades de transposasas (Bi et al., 2019). Las cuatro AbaR más estudiadas son AbaR0/AbaR3, que contienen los genes de resistencia a antibióticos *sul1*, *tetA(A)*, *catA1*, bla_{TEM}, *aphA1b*, *aaC1*, *aadA1* (Hamidian and Hall, 2018); AbaR4, asociada a $bla_{\text{OXA-23}}$ (Kim et al., 2012), AbaGRI1, que contiene a los genes $bla_{\text{OXA-23}}$, *sul2*, *tetA(B)*, *tetR(B)*, *strA* y *strB* (Nigro and Hall, 2016); y AbaGRI2, que contiene los genes bla_{TEM}, *aphA1b*, *catA1*, *sul1*, *aadA1*, y *aacC1* (Blackwell et al., 2016). Cada tipo de isla de resistencia deriva de la combinación de uno o más transposones distintos. AbaR0/AbaR3 y AbaGRI1 suelen insertarse en medio del gen *comM*, que codifica para una ATPasa, pero también pueden insertarse en otras regiones (Holt et al., 2016; Bi et al., 2019). Además, AbaR0/AbaR3 y AbaR4 están asociadas a los linajes 1 y 2 de la GC1, mientras que AbaGRI1 y AbaGRI2 se asocian con la GC2 (Holt et al., 2016). Si bien las islas tipo *AbaR* son muy prevalentes en las clonas internacionales 1 y 2 de *A. baumannii*, también hay algunos reportes de estas islas en *A. nosocomialis* y *A. seifertii* (Kim et al., 2012; Kim and Ko, 2015). Estos elementos genéticos usualmente se encuentran en el cromosoma, y pocas

veces en plásmidos, lo cual pudiera explicar su baja frecuencia fuera de *A. baumanii* (Bi et al., 2019).

También existen los integrones, que son plataformas de expresión y de adquisición de genes (Gillings, 2014; Partridge et al., 2018). Entre los genes que más los conforman están los de resistencia a los antibióticos, a los desinfectantes, y a algunos metales. Estos elementos portan varios genes de resistencia y son transmisibles por transferencia genética horizontal. Se han identificado integrones en *A. baumannii*, *A. nosocomialis*, *A. pittii*, *A. johnsonnii*, y *A. junii* asociados a resistencia a múltiples antibióticos como carbapenémicos, sulfonamidas, y aminoglucósidos (Yamamoto et al., 2011; Kim et al., 2013; Martins et al., 2015).

Genes de resistencia a antibióticos

Parte de lo que guía el tratamiento para combatir infecciones bacterianas es la especie a la que pertenece el microorganismo y su perfil de resistencia a antibióticos. El diagnóstico preciso de a qué especie pertenece un aislado determinado, ayuda a guiar la terapia antibiótica, pues hay algunas bacterias con mecanismos de resistencia intrínseca, por lo que ciertos antibióticos nunca van a ser efectivos contra ellas (Fang and Abbott, 2015). Tomemos como ejemplo el caso en el que un antibiótico sea útil contra bacterias Gram-positivas, pero si el antibiótico no puede entrar a la célula porque no puede atravesar las dos membranas de una bacteria Gram-negativa, el fármaco no va a ser efectivo en estas últimas (Peterson and Kaur, 2018). Asimismo, las bacterias pueden adquirir determinantes de resistencia a antibióticos, que les confieren inmunidad ante moléculas que de otra forma sí las podrían afectar (Partridge et al., 2018). Los antibióticos interfieren con algún proceso biológico importante o atacan alguna estructura celular, y los mecanismos de resistencia consisten en evitar que el antibiótico llegue a su objetivo, ya sea por permeabilidad diferencial que evita que la molécula pueda entrar, disminución de la concentración del antibiótico por flujo activo del mismo hacia el exterior, por cambios en el sitio blanco; o bien, puede haber modificaciones enzimáticas que impidan que el antibiótico tenga actividad (Walsh and Wencewicz, 2015a; Shin and Park, 2017).

Resistencia intrínseca a los antibióticos

La resistencia intrínseca a la daptomicina, a las estreptrograminas, a los glucopéptidos (como la vancomicina), a las lincosamidas, y a los macrólidos (como la eritromicina), se debe a que estos antibióticos, de diversos mecanismos de acción, no pueden atravesar las dos membranas de las bacterias Gram-negativas (Abbott et al., 2013; Walsh and Wencewicz, 2015a). La resistencia intrínseca a la ampicilina y a las cefalosporinas de primera y segunda generación (como la cefalotina, la cefazolina, la cefuroxima, y la cefoxitina), beta-lactámicos que interfieren con la síntesis de la pared celular, se debe a la presencia de beta-lactamasas cromosomales, principalmente oxacilinasas, y en algunas especies como *A. baumannii* a una cefalosporinasa tipo AmpC, pues su producción basal es suficiente para que degraden a esos antibióticos (Poirel et al., 2011; Ruppé et al., 2015; Lupo et al., 2018).

Las variantes genéticas de las oxacilinasas (*bla*$_{\text{OXA}}$) intrínsecas están relacionadas con la especie o con el clado, por lo que a veces se han utilizado como guía en la identificación de las especies. Por ejemplo, la *bla*$_{\text{OXA-51}}$ está asociada con *A. baumannii*, mientras que las *bla*$_{\text{OXA-214}}$ y *bla*$_{\text{OXA-215}}$ están asociadas con *A. haemolyticus* (Figueiredo et al., 2012). Sin embargo, estos genes no están exentos de la transferencia genética horizontal, y pueden encontrarse en distintas especies, por lo que no se recomienda su uso como marcador taxonómico (Evans and Amyes, 2014).

Resistencia a los antibióticos por la pérdida de porinas

Las porinas son proteínas de la membrana externa que forman canales por los cuales pueden pasar moléculas de distintos tamaños, dependiendo del diámetro del poro (Abbott et al., 2013). Si no se expresa el gen que codifica para la proteína de membrana externa asociada a carbapenémicos, del inglés *carbapenem associated outer membrane protein*, CarO, la bacteria puede ser resistente a los carbapenémicos, otro tipo de beta-lactámicos, (Abbott et al., 2013), pero con el costo de no poder importar algunos aminoácidos (Shin and Park, 2017).

Resistencia a los antibióticos por bombas de eflujo

Las bombas de eflujo disminuyen de manera activa la concetración de antibiótico en la célula. Éstas se dividen en distintas familias; pero las más importantes para la resistencia a antibióticos en el género *Acinetobacter* son las bombas tipo RND y MFS. Las bombas tipo RND reciben su nombre por los fenotipos con los que se relacionan en distintas bacterias: resistencia, nodulación y división. En los genomas del género *Acinetobacter* existe al menos un operón que codifica para este tipo de bombas, entre ellos los operones *ade*, del inglés ***A****cinetobacter* ***d****rug* ***e****fflux*, *adeABC*, *adeFGH*, y *adeJIK* (Poirel et al., 2011; Abbott et al., 2013). La sobre-expresión de estas bombas confieren resistencia a una amplia variedad de antibióticos, tales como los beta-lactámicos, las fluoroquinolonas (que interfieren con la replicación del ADN), los aminoglucósidos, el cloranfenicol, y las tetraciclinas (los cuales interfieren con la síntesis de proteínas) (Vila et al., 2007). Las bombas más representativas del tipo MFS, del inglés ***M****ajor* ***F****acilitator* ***S****uperfamily*, son las que confieren resistencia a la tetraciclina. Los genes *tet* codifican para estas bombas, se activan ante la presencia de la tetraciclina, y suelen estar en transposones (Vila et al., 2007).

Resistencia a los antibióticos por cambios en el sitio blanco

La acción de algunos antibióticos depende del correcto acomodo de la molécula de antibiótico con el sitio blanco que tengan en la maquinaria celular. Así que, si hay cambios en el sitio blanco que impidan que se forme el complejo con el antibiótico, la bacteria será inmune a ese fármaco.

Las mutaciones en *gyrA* y *parC*, los genes de la maquinaria basal que codifican para una de las subunidades que componen a la DNA girasa y una topoisomerasa, respectivamente, disminuyen la afinidad de la proteína blanco hacia las quinolonas (Poirel et al., 2011); de esta manera, la síntesis del ADN no se afecta y la bacteria puede sobrevivir.

El gen *armA* codifica para una metil-transferasa que modifica al 16S rRNA. Dicha modificación evita que los aminoglucósidos interfieran con la síntesis de proteínas, pues ya no pueden reconocer al sitio A del ribosoma (Doi et al., 2016; Lupo et al., 2018). Este gen se ha encontrado en algunos transposones asociados a plásmidos y al cromosoma de algunas cepas de *A. baumannii* (Karah et al., 2016; Blackwell et al., 2017).

Las polimixinas como la colistina (también conocida como polimixina E) y la polimixina B desestabilizan la membrana externa de las bacterias porque tienen carga positiva e interactúan con la carga negativa del lipo-oligosacárido, uno de los principales componentes de la membrana externa de las bacterias del género *Acinetobacter* (Walsh and Wencewicz, 2015c). Las mutaciones de pérdida de función de los genes cromosomales que codifican para la síntesis del lípido A (*lpxA, lpxC, lpxD*, y *lpsB*), uno de los principales componentes del lipo-oligosacárido, confiere resistencia a estos antibióticos. Sin embargo, este tipo de mutaciones pueden comprometer el crecimiento y la estabilidad de la membrana de la bacteria (Jeannot et al., 2017; Lima et al., 2018). La adquisición mediada por plásmidos de variantes del gen *mcr*, que modifica al lípido A (Hameed et al., 2019; Ma et al., 2019), así como las mutaciones en los genes cromosomales *pmrA* o *pmrB* que desencadenan la sobreexpresión de otras enzimas que modifican al lípido A (PmrC, NaxD), también confiere resistencia a las polimixinas, pero sin comprometer la viabilidad ni la virulencia de la bacteria (Jeannot et al., 2017; Lima et al., 2018).

Resistencia a los antibióticos por modificaciones enzimáticas y degradación del antibiótico

Las modificaciones enzimáticas o la degradación de los antibióticos evitan que éstos ejerzan su acción. Las enzimas que modifican a los aminoglucósidos pueden ser fosfo-transferasas (codificadas por los genes *aph* y *str*), acetil-transferasas (codificadas por los genes *aac*), o nucleotil-transferasas (codificadas por los genes *aad* y *ant*), y los genes que las codifican pueden encontrarse tanto en plásmidos como en el cromosoma, usualmente asociados a transposones o a integrones (Becker and Cooper, 2013; Karah et al., 2016; Blackwell et al., 2017). Las modificaciones enzimáticas impiden que los aminoglucósidos interactúen con la subunidad pequeña del ribosoma (30S), por lo que la traducción de proteínas no se afecta (Walsh and Wencewicz, 2015b).

Las beta-lactamasas hidrolizan a los beta-lactámicos, por lo que la síntesis de la pared celular no se debilita, ni se lisa la bacteria. Si se sobre-expresan las beta-lactamasas intrínsecas, entonces puede haber una alta resistencia con relevancia clínica; además algunas mutaciones amplían la gama de sustratos que pueden hidrolizar estas enzimas

(Evans and Amyes, 2014). Existen diversas familias de beta-lactamasas; la clasificación más utilizada se basa en homología (la clasificación molecular) que las divide en las familias A, B, C, y D (Bush, 2013). Las beta-lactamasas de clase A hidrolizan penicilina; pero ciertas mutaciones amplían su rango de hidrólisis (por lo que se denominan beta-lactamasas de espectro extendido, del inglés *ESBL*), y se encuentran asociadas a plásmidos, como las bla_{CTX} que confieren resistencia cefalosporinas; sin embargo, este tipo de enzimas no es tan frecuente en el género *Acinetobacter* (Walther-Rasmussen and Høiby, 2004; Poirel et al., 2008; Bush, 2013; Ghafourian et al., 2015; Zago et al., 2016). Las beta-lactamasas de clase B utilizan un ión metálico como cofactor, por lo que se denominan metalo-beta-lactamasas. La metalo-beta-lactamasa (MBL) $bla_{\text{NDM-1}}$ es el representante más problemático de esta familia, pues su rango de acción incluye a las penicilinas, a las cefalosporinas, y a los carbapenémicos. Este tipo de enzimas suele encontrarse en transposones asociados al cromosoma y a plásmidos (Bush, 2013); pero también hay otras MBLs asociadas a integrones (Gupta, 2008). Las beta-lactamasas de clase C degradan cefalosporinas y sus genes se encuentran en el cromosoma; su sobre-expresión suele deberse a la presencia de un promotor fuerte, en la orientación correcta, ligado a secuencias de inserción (Bush, 2013; Ghafourian et al., 2015). Las beta-lactamasas de clase D se caracterizan por hidrolizar oxacilina, de ahí su nombre de oxacilinasas (bla_{OXA}), pero algunas mutaciones les permiten hidrolizar carbapenémicos; este tipo de enzimas se encuentran codificadas en el cromosoma y en plásmidos (Bush, 2013; Evans and Amyes, 2014; Ghafourian et al., 2015).

Factores de virulencia

Los factores de virulencia son “componentes microbianos que causan daño al hospedero” (Casadevall and Pirofski, 2003), entre los cuales encontramos diversas toxinas, factores de adherencia, distintos sistemas de secreción, de adquisición de nutrientes (Dobrindt et al., 2004), y mecanismos de defensa o de protección contra el sistema inmune del hospedero (Rosenstein and Götz, 2012). El daño al hospedero puede derivarse de la acción concreta del factor de virulencia, como las hemolisinas que lisan a los eritrocitos, o bien, de la respuesta exacerbada del sistema inmune que puede causar incluso la muerte del hospedero,

como sucede con los superantígenos de *Streptocccus pyogenes* (Strus et al., 2017; Healy and Baker, 2018). Además, hay diferencias en la virulencia entre distintas cepas, así como entre distintas especies del género *Acinetobacter*, lo cual pudiera deberse al contenido de genes, diferencias en la expresión, y al estado del hospedero (de Breij et al., 2012; Peleg et al., 2012; Tayabali et al., 2012; Skerniškytė et al., 2019).

Podemos agrupar por su función a los factores de virulencia del género *Acinetobacter* en proteínas que permiten evadir al sistema inmune, factores adherencia, cápsulas y sistemas de adquisición de micronutrientes. Tan sólo los *loci* de la cápsula y de algunos sistemas de captura y aprovechamiento de hierro (sideróforos) se agrupan en regiones genómicas específicas mientras que el resto de los factores de virulencia suele encontrarse en distintas regiones del cromosoma (Eijkelkamp et al., 2011; Singh et al., 2019).

Proteínas que le causan algún daño concreto al hospedero

El daño al hospedero puede estar mediado por la acción concreta de enzimas de la bacteria, tales como las fosfolipasas y las proteasas. La producción de estas proteínas, si es que se excretan, se puede revisar en medios de cultivo sólido suplementado con gelatina (para detectar gelatinasas, un tipo de proteasa) o con sangre (para detectar hemolisinas, un tipo de fosfolipasas) (Poh and Loh, 1985). Otra manera de identificar la producción de este tipo de proteínas es por medio de la cuantificación del RNA mensajero bajo distintas condiciones (Stahl et al., 2015; Fiester et al., 2016), o mediante el uso de anticuerpos (Kinsella et al., 2017).

Las fosfolipasas rompen a los fosfolípidos, lo cual puede desestabilizar la membrana de las células del hospedero y que terminen por lisarse. En algunas cepas de *A. baumannii* se encontraron fosfolipasas que se expresan en condiciones de bajo hierro y que desencadenan la lisis de eritrocitos (Fiester et al., 2016), lo cual les puede ayudar a adquirir el hierro por medio de distintos sistemas. En contraste, existen otras cepas de *A. baumannii* que son capaces de usar un fosfolípido característico de las células de humanos (la fosfatidilcolina) como única fuente de carbono, y contienen otras fosfolipasas (Stahl et al., 2015).

Las proteasas son enzimas que rompen proteínas, y se pueden dividir por el tipo de sustrato que degraden. Por ejemplo, existen las colagenasas que degradan fibras de colágeno, componentes de las células humanas que le dan estructura y resistencia a diversos tejidos, y que se encuentran en patógenos como *Clostridium histolyticum* (Duarte et al., 2016), pero todavía no hay reportes confiables de la presencia de estas enzimas en el género *Acinetobacter*. Sin embargo, en algunas especies de *Acinetobacter*, hay gelatinasas, enzimas que son capaces de degradar al colágeno desnaturalizado (Duarte et al., 2016; Sedo et al., 2016), pero todavía no se ha caracterizado su rol en la infección por estos organismos. Por otro lado, en *A. baumannii*, *A. nosocomialis* y en *A. pittii* se identificó a la proteasa CpaA que interfiere con la coagulación de la sangre porque rompe a uno de los factores de coagulación (Kinsella et al., 2017; Waack et al., 2018), así como a la proteasa CipA, caracterizada en *A. baumannii* (Koenigs et al., 2016), que también interfiere con la coagulación. Este mecanismo le da ventaja a las bacterias para que se disemine la infección y no actúe el sistema inmune, pues la formación de un coágulo en las heridas es primordial para la acción de las células blancas del hospedero.

Proteínas para la evasión del sistema inmune del hospedero

Una de las primeras respuestas del sistema inmune del hospedero es la producción de especies reactivas de oxígeno, específicamente por medio de los neutrófilos, que son las primeras células en llegar al sitio de infección; posteriormente, la producción de distintas moléculas, ya sean de origen bacteriano o del hospedero, puede llamar a otras células del sistema inmune (García-Patiño et al., 2017). Las moléculas bacterianas que interfieran con alguno de esos procesos ayudan a la evasión del sistema inmune. Entre estas proteínas encontramos a las catalasas (Sun et al., 2016) y a las superóxido-dismutasas que pudieran proteger a las bacterias de las especies reactivas de oxígeno; empero, su papel en las infecciones se ha caracterizado poco (Heindorf et al., 2014; Sato et al., 2019). Asimismo, se han reportado como responsables en la evasión de la respuesta inmune a las proteasas que interfieren con la coagulación (como CipA y CpaA, previamente mencionadas) y a las proteínas que permiten la invasión de las células del hospedero (descritas en la siguiente sección).

Factores de adherencia

Los factores de adherencia le ayudan a las bacterias a unirse a las superficies inertes o a las células del hospedero. La capacidad de unirse a superficies le da ventajas a patógenos en los hospitales porque les permite permanecer en plástico y otros materiales del equipo médico, mientras que la adhesión a las células del hospedero les permite a las bacterias invadirlas si cuentan con mecanismos adicionales para hacerlo.

OmpA es la proteína de superficie más abundante en *A. baumannii*, es muy similar entre distintas cepas, y todas las especies del género *Acinetobacter* cuentan con homólogos de esta proteína (Morris et al., 2019). En el caso de *A. baumannii*, OmpA puede inducir la apopotosis de las células del hospedero (Choi et al., 2005); pero también es importante para que las bacterias puedan adherirse a las células epiteliales, y en algunos casos para invadirlas (Gaddy et al., 2009; Smani et al., 2012; García-Patiño et al., 2017), así como para la formación de biopelículas (Gaddy et al., 2009).

La proteína Bap de *A. baumannii* permite que las bacterias formen biopelículas estables, y en algunas cepas, también sobre superficies de relevancia médica, tales como kits y bolsas de nutrición parenteral hechas de poliestireno y de polipropileno, y sobre algunos instrumentos quirúrgicos y prótesis ortopédicas hechas de titanio (Loehfelm et al., 2008). Además, algunas cepas de *A. baumannii* utilizan a *Bap* para adherirse a las células humanas *in vitro* (Brossard and Campagnari, 2012).

La proteína Ata permite la adherencia de algunas cepas del género *Acinetobacter* a las células humanas *in vitro*, y está presente en la mayoría de los genomas secuenciados de *A. baumannii* y en menor proporción en otras especies cercanas a ésta como *A. calcoaceticus* y *A. pittii* (Rahbar et al., 2019; Weidensdorfer et al., 2019). En contraste, la proteína *AtaA* del aislado ambiental *Acinetobacter sp.* Tol5, permite la adherencia a superficies de poliestireno y de acero inoxidable, un material del cual está hecha la mayoría del instrumental quirúrgico (Ishikawa et al., 2012; Koiwai et al., 2016).

Cápsula

La cápsula es una capa formada por repeticiones de oligosacáridos que envuelve a la bacteria, y que puede conferirles distintas ventajas, tales como la resistencia desecación para sobrevivir por largos períodos de tiempo en superficies inertes, la capacidad de evadir al sistema inmune del hospedero, e incluso la resistencia a algunos agentes antimicrobianos, pues funge como una barrera extra entre la bacteria y el ambiente (Geisinger et al., 2019; Singh et al., 2019). En el género *Acinetobacter*, los azúcares que conforman a la cápsula varían mucho porque dependen de la variedad de enzimas presentes en los *loci* de biosíntesis de la cápsula (KL) y de la biosíntesis de los oligosacáridos de la parte externa (OCL), los cuales son altamente variables entre cepas (Shashkov et al., 2016; Geisinger et al., 2019; Singh et al., 2019).

Los *loci* KL son regiones largas, de entre 20 y 35 kilobases, que en *A. baumannii* están flanqueadas por los genes *fkpA* y *lldP*, mientras que los *loci* OCL están flanqueados por los genes *ilvE* y *aspS;* estos patrones a veces permiten la identificación y delimitación de dichas regiones (Kenyon and Hall, 2013; Singh et al., 2019). En los *loci* KL se encuentran las enzimas para la síntesis de los oligosacáridos que componen a la cápsula, para su polimerización, y para su exportación hacia la superficie de la membrana externa (Geisinger et al., 2019; Singh et al., 2019). En los *loci* OCL hay enzimas involucradas en la síntesis del "andamio molecular" a la que se le añaden los oligosacáridos, el *core* externo del lípido A, que se ancla a la membrana externa y está formado por azúcares (glucosamina), grupos fosfato, y cadenas de ácidos grasos (Kenyon and Hall, 2013; Steimle et al., 2016; Singh et al., 2019).

Sistemas de adquisición de hierro

El hierro es un micronutriente necesario en cantidades muy pequeñas, esencial para llevar a cabo reacciones enzimáticas. Los sistemas de adquisición de hierro le permiten a las bacterias evadir la "inmunidad por nutrientes", que consiste en que el sistema inmune del hospedero limite la disponibilidad de estos elementos, por su captura mediada por proteínas, para limitar o evitar el crecimiento bacteriano (Mortensen and Skaar, 2013; Morris et al., 2019).

Los transportadores FeoAB importan hierro ferroso (Fe^{2+}) al interior de la célula (Mortensen and Skaar, 2013). Los sideróforos son moléculas con alta afinidad por el hierro férrico (Fe^{3+}), el estado de oxidación preferido por las células a pesar de ser poco soluble (Lee et al., 2017). Los sideróforos compiten por el hierro con las células del hospedero para su captura y aprovechamiento. Entre los sideróforos caracterizados en *Acinetobacter* se encuentran la acinetobactina, la baumanoferrina, y la enterobactina (Dorsey, 2003). La acinetobactina se sintetiza por las proteínas codificadas por los genes *basA*, *basB*, *basC*, *basD*, *basF*, *basG*, *basH*, *basI*, y *basJ* (Zimbler et al., 2009; Hasan et al., 2015); las proteínas BfnA, BfnB, BfnD, BfnE, BfnG, y BfnL están involucradas en la síntesis de la baumanoferrina (Penwell et al., 2015); y las proteínas DhbA, DhbB, y DhbE sintetizan a la enterobactina (Mortensen and Skaar, 2013).

Antecedentes

Hay reportes esporádicos de *A. haemolyticus* clínicas asociadas a infecciones oportunistas. Podemos dividir esos reportes en aquellos con asignación taxonómica con evidencias débiles y en los que se basan en evidencias confiables, para las distintas cepas clínicas bajo estudio. En el primer caso (Tabla 1) hay un promedio de 7 cepas por período de estudio, desviación estándar de 12, y mediana de 2 cepas; el valor tan alto de la desviación estándar se debe a que hay algunos estudios que reportan decenas de cepas en un período desde un año (Astal and El Astal, 2004; El Astal, 2005; Saha and Rit, 2012), 4 años (Traub and Spohr, 1989), o hasta 10 años (Ramette and Kronenberg, 2018). Mientras tanto, en las evidencias confiables para la asignación taxonómica (Tabla 2) tenemos un promedio de 4 cepas por período de estudio, desviación estándar de 3, y mediana de 3 cepas; aquí los valores son mucho más bajos, y el reporte con más cepas (10) abarca un período de 4 años (Gundi et al., 2009). En contraste, en dos hospitales de México también encontramos más de 10 aislados en unos períodos de 6 y 8 años. En el Instituto Nacional de Cancerología, del 2008 al 2015 hubo 11 cepas, mientras que el Hospital para el Niño Poblano hubo 17 cepas entre 2010 y 2016. Además, a nivel nacional, a partir de 2014, aumentó la incidencia de infecciones por *A. haemolyticus* en México (Secretaría de Salud, 2016). Hasta 2009 todos los reportes de evidencias confiables de infecciones por *A. haemolyticus* en el mundo eran sobre cepas sensibles a la mayoría de los antibióticos. Entre 2009 y 2010 se encontró en China una cepa que portaba en un plásmido a la metalo-beta-lactamasa NDM-1 (Fu et al., 2012), que confiere resistencia a carbapenémicos, uno de los medicamentos de última elección para el tratamiento de infecciones por *Acinetobacter*.

El aumento en la cantidad de reportes de infecciones causadas por *A. haemolyticus* pudiera deberse a que las cepas actuales son más patogénicas que antes, o bien, a que apenas hace unos pocos años se han podido diferenciar de otras especies con el uso de métodos más precisos de genotipificación. Sin embargo, resulta importante conocer la magnitud del problema que representa *A. haemolyticus*, pues no sólo ha habido aumentos en los reportes de infecciones de *A. haemolyticus*, sino también ya hay reportes de adquisición de determinantes de resistencia de preocupación global (como la metalo-beta-lactamasa NDM-

1)- Además, no hay datos publicados sobre la diversidad de aislados asociados a infecciones por *A. haemolyticus* ni sus perfiles de resistencia.

Tabla 1. Reportes de *Acinetobacter haemolyticus* clínicas - Evidencias débiles

Referencia	**Período de estudio**	**País**	**Métodos de identificación**	**Susceptibilidad o resistencia a antibióticos**	**Número de aislados de *A. haemolyticus* de un total de N aislados del género *Acinetobacter***
(Merkier and Centrón, 2006)	1982 – 2005	Argentina	ARDRA Pruebas bioquímicas Secuencia parcial del 16S ribosomal	*bla*$_{\text{OXA-51}}$-like negativa	2 / 200
(Traub and Spohr, 1989)	1985 – 1989	Alemania	API 20E mini	"Susceptibles a gentamicina, ciprofloxacino, sulbactam, ofloxacina, y ampicilina-sulbactam Resistentes a amikacina, netilmicina, tobramicina"	12 / 144
(Seifert et al., 1993)	1991 – 1992	Alemania	Pruebas bioquímicas	"Susceptibles a cefotaxima, ceftazidima, ceftriaxona, aztreonam, imipenem, gentamicina y ciprofloxacino"	3 / 180
(Castellanos Martínez et al., 1995)	1994	España	API ID 32GN	"Susceptible a ampicilina, cefalosporinas de tercera generación, aminoglucósidos e imipenem"	1
(Iglesias de Sena et al., 2004)	1998	España	Pasco (Difco)	"Susceptible a aminoglucósidos, quinolones, algunas cefalosporinas (cefotaxima, ceftazidima y cefepime), y todos	1

				los betalactámicos excepto ampicilina Resistente a algunas cefalosporinas (cefalozina, cefoxitina, cefuroxime), trimetoprima con sulfametoxazol, nitrofurantoina, cloranfenicol, y fosfomicina”	
(Prashanth and Badrinath, 2006)	1998 – 2000	India	Pruebas bioquímicas	No hay información	2 / 49
(Grotiuz et al., 2006)	2001	Uruguay	Pruebas bioquímicas	“Susceptibles a piperacilina, ceftazidima, cefepime, imipenem, gentamicina, amikacina, ciprofloxacino, trimetoprima con sulfametoxazol, y tetraciclina”	2
(Quinteira et al., 2007)	2001 – 2004	Portugal	Secuencia parcial del 16S ribosomal	“Resistentes a ciprofloxacino, colistina y a todos los betalactámicos, excepto cefepima, ceftazidima, y aztreonam; la resistencia a aminoglucósidos era variable”	2 / 224 imipenem resistant *Acinetobater*
(Astal and El Astal, 2004)	2003	Palestina	API 20E	“Resistentes a amoxicilina, doxiciclina, y trimetoprima con sulfametoxazol”	51 / 51
(El Astal, 2005)	2004	Palestina	API 20E	“Resistentes a amoxicilina, trimetoprima con	18 / 18

				sulfametoxazol doxiciclina, cefalexina, gentamicina, y ácido nalidíxico"	
(Ramette and Kronenberg, 2018)	2005 - 2016	Suiza	NA	"Susceptibles a antibióticos"	12/632
(Chatterjee et al., 2016)	2007 – 2014	India	ARDRA MiniAPI Vitek2	"Susceptible a minociclina and tigeciclina. Resistente a ceftazidima, cefepime, aztreonam, doripenem, imipenem, meropenem, amikacina, gentamicina, ciprofloxacino" *bla*$_{\text{NDM-1}}$ positiva *armA* positiva *intI1* positiva	1 / 68
(Saha and Rit, 2012)	2010 – 2011	India	Pruebas bioquímicas	No hay información	12 / 154
(Jhingan et al., 2018)	2010 – 2015	India	Pruebas bioquímicas Vitek2	"Susceptibles a amikacina y gentamicina Probablemente eran resistentes a ceftazidima"	2 / 11
(Jones et al., 2015)	2012	Paquistán	MALDI-TOF Secuencia parcial del 16S ribosomal	*bla*$_{\text{NDM-1}}$ positiva	3
(Dirección de Redes en Salud Pública and Subdirección Laboratorio Nacional de Referencia, 2014)	2012 – 2014	Colombia	NA	*bla*$_{\text{NDM-1}}$ positiva	1 / 4

(Lowe et al., 2018)	2013 – 2014	Sudáfrica	MALDI-TOF	*bla*$_{OXA-51}$-like negativa	1 / 141
(Al Bshabshe et al., 2016)	2014 – 2015	Arabia Saudita	Vitek2	"Susceptibles a amikacina, y colistin; sólo 1 cepa era susceptible a imipenem La mayoría eran multi-drogo-resistentes"	4 / 105
(Secretaría de Salud, 2016)	2015	México	NA	No hay información	No hay información
(Secretaría de Salud, 2016)	2015	México	NA	No hay información	10 / 87
(Parra-Flores et al., 2017)	2015	México	NA	No hay información	1
(Sáenz-Talavera et al., 2017)	2015 – 2016	México	Vitek2	"Multi-drogo-resistentes"	1 / 2

Tabla 2. Reportes de *Acinetobacter haemolyticus* clínicas - Evidencias confiables

Referencia	**Período de estudio**	**País**	**Métodos de identificación**	**Susceptibilidad o resistencia a antibióticos**	**Número de aislados de *A. haemolyticus* de un total de N aislados del género *Acinetobacter***
(Gundi et al., 2009)	2001 – 2005	Francia	Secuencia parcial de *rpoB*	No hay información	10 / 99
(Schleicher et al., 2013)	2005 - 2009	Alemania	ARDRA Secuencia parcial de *rpoB*	Susceptibles a imipenem	3 / 376
(Ko et al., 2007)	2006	Corea del Sur	Secuencia parcial de *rpoB*	Susceptibles a polimixinas	? / 159
(Turton et al., 2010)	2008 – 2009	Reino Unido y República de Irlanda	Secuencia parcial de *rpoB*	"Susceptibles a antibióticos"	2 / 690
(Wang et al., 2014)	2009 – 2010	China	MALDI-TOF, Secuencia parcial del 16S ribosomal Secuencia parcial de *rpoB* Vitek2	"Susceptibles a antibióticos $bla_{\text{OXA-51}}$-like negativa	2 / 385
(Fu et al., 2012)	2009 – 2010	China	Secuencia parcial de *rpoB* Vitek2	Resistente a carbapenémicos y cefalosporinas de amplio espectro $bla_{\text{NDM-1}}$ positiva	1 / 9
(Jeong et al., 2016)	2012 – 2015	Corea del Sur	MALDI-TOF Secuencia parcial del 16S ribosomal Secuencia parcial de *rpoB* Vitek2	No hay información	3 / 729

Hipótesis

Si *Acinetobacter haemolyticus* ya está en el proceso de adquirir genes de algunas bacterias que se encuentran adaptadas al ambiente del hospital, entonces es posible que *A. haemolyticus* se convierta en un problema de salud tan grave como ya lo es *A. baumannii*. Entre los genes que podría adquirir se encuentran factores de virulencia, genes de resistencia a antibióticos y a desinfectantes.

Objetivo general

Determinar si *A. haemolyticus* se en encuentra en el proceso de convertirse en un problema de salud tan grave como ya lo es *A. baumannii*.

Objetivos particulares

- Obtener un conjunto de genomas de *Acinetobacter haemolyticus* para responder la pregunta de investigación, incluyendo cepas mexicanas y de la base de datos de RefSeq. Secuenciar por Illumina a las cepas mexicanas elegidas.

- Asegurarme de que los genomas seleccionados son de la especie con la que espero trabajar, y filtrar el conjunto de datos por su calidad.

- Hacer análisis de genómica comparativa entre todas las cepas (mexicanas y de RefSeq) y contra bases de datos de genes de resistencia, factores de virulencia, elementos genéticos móviles, y otros elementos genéticos que pudieran estar involucrados en la supervivencia y proliferación de *A. haemolyticus* en los hospitales.

- Si las cepas no son clonales, secuenciar por PacBio al menos una cepa representante de cada clado. En caso de que todas sean clonales, elegir una sola cepa para secuenciarla por PacB io y cerrar su genoma.

- Subir cada uno de los mejores ensambles de las cepas mexicanas a NCBI.

- Hacer análisis de arquitectura genómica y análisis de contenido génico más detallado con las anotaciones de NCBI.

Materiales y métodos

Para obtener el conjunto de cepas objeto de mi análisis, primero descargué los genomas de *A. haemolyticus* disponibles en la base de datos de RefSeq del NCBI, del inglés *National Center for Biotechnology Information*. Además, dado que hasta el momento del inicio del proyecto no había ninguna cepa mexicana secuenciada – y que por antecedentes con trabajos con *A. baumannii* encontramos que las bacterias presentes en hospitales mexicanos no suelen ser las mismas que los de otros países— elegí un conjunto de cepas mexicanas para enviar a secuenciar. Escogí a las cepas mexicanas con una aproximación a que se trate de distintas cepas con los siguientes criterios: que fueran aisladas en hospitales en distintos años (1998, 2008, 2011-2015), de diferentes pacientes y con distinto contenido de plásmidos. En los hospitales en los que se aislaron las cepas, como parte de sus protocolos de rutina, se hicieron pruebas para identificar la posible especie a la que pertenecían los aislados, tales como pruebas bioquímicas como API de Biomerieux, Vitek2, o la espectrometría de masas (MALDI-TOF). Sin embargo, para corroborar dichos resultados, purifiqué el ADN genómico de las cepas, amplifiqué y cloné una porción del gen *rpoB* (Gundi et al., 2009), y lo mandé a secuenciar. Descarté aquellas cepas cuya secuencia no indicara que se tratara de *A. haemolyticus*. Posteriormente, elegí al azar cepas de distintos hospitales (dentro de un mismo hospital elegimos entre aquellas que tengan un perfil de plásmidos distinto) para nuevamente purificar ADN genómico y enviarlo a secuenciar por Illumina.

Ensamblé y anoté los genomas de las cepas mexicanas secuenciadas por Illumina; reanoté las cepas de RefSeq para que todos los ensambles estuvieran anotados con la misma herramienta (Prokka (Seemann, 2014)).

Para comprobar la especie a la que pertenecían las primeras versiones de los ensambles de las cepas mexicanas y de los ensambles disponibles en RefSeq, usé la identidad nucleotídica promedio (ANIm) (Pritchard et al., 2016), así como filogenias con genes que

codifican para proteínas ribosomales. Descarté los ensambles que no correspondían con *A. haemolyticus*, así como los ensambles de mala calidad y los distintos ensambles de la misma cepa.

Hice análisis de genómica comparativa con los genomas de todas las cepas. Además, verifiqué si los genomas contenían genes cuyo producto confiere resistencia a antibióticos o resistencia a metales, genes que codificaran para factores de virulencia, o elementos genéticos móviles distintos a los plásmidos; por ejemplo, fagos y transposones.

Dados los resultados de los análisis de genómica comparativa, elegí al azar algunas cepas mexicanas de cada clado para terminar sus genomas con lecturas largas. Purifiqué el ADN genómico de dichas cepas y lo envié a secuenciar por PacBio para poder hacer ensambles híbridos. Después, mejoré los ensambles de las cepas mexicanas y subí el mejor ensamble para cada cepa a NCBI.

Descargué los genomas de las cepas mexicanas anotadas por NCBI y, dado que los genomas en RefSeq fueron anotados con la misma herramienta que los de las cepas mexicanas ("NCBI Prokaryotic Genome Annotation Pipeline", PGAP), usé las anotaciones de NCBI para rehacer los análisis de genómica comparativa, hacer los análisis de arquitectura genómica y los análisis de contenido génico que se detallan en el artículo.

Resultados

Ensamblé y anoté los genomas de 32 cepas mexicanas de A. haemolyticus, y cerré el cromosoma de 9 de esas cepas. De los genomas de NCBI, descarté dos que no pertenecían a la especie A. haemolyticus (JKSF06 y KCRI-45), uno que era de mala calidad (TG19602), y otros que se referían a la misma cepa -conservé el que está en negritas- (**CIP 64.3** = MTCC 9819 = NBRC 109758 = TG19599 y **ATCC 27244** = TG21157).

Analicé los genomas de, en total, 44 cepas de *A. haemolyticus*, los cuales indican que la especie tiene un pangenoma abierto. La distribución de las cepas mexicanas y las cepas chinas en los clados del árbol filogenético obtenido por genes ortólogos monocopia sin señales de recombinación, indica que hay distintos linajes circulando en los hospitales de ambos países. Tanto el árbol filogenético como las variantes de un solo nucleótido, del inglés SNV, señalan que las cepas del mismo país se parecen más entre sí que con las de otros países.

El cromosoma de *A. haemolyticus* se divide en 12 regiones donde se conserva el orden relativo de los genes, es decir, son regiones sinténicas, y es en esas regiones donde se encuentra la mayoría de los genes compartidos entre todas las cepas (los genes *core*). Las regiones sinténicas están separadas por regiones altamente variables (regiones hipervariables), tanto en tamaño como en contenido génico. Algunas regiones hipervariables están compartidas entre cepas, y otras son únicas, a pesar de encontrarse entre las mismas regiones sinténicas, es decir, compartir el mismo contexto. Además, las regiones hipervariables tienen señales de transferencia genética horizontal, del inglés HGT, como señales de recombinación, composición nucleotídica anómala, genes únicos, y componentes de elementos móviles como transposasas y genes de fagos.

Respecto al contenido de genes de resistencia a antibióticos, todas las cepas, tanto mexicanas como de NCBI, contienen un gen de resistencia a aminoglucósidos en la misma región, y todas las cepas tienen un fragmento o el gen completo que codifica para una OXA-214 u OXA-215 (característica de la especie *A. haemolyticus* (Evans and Amyes, 2014)). Varias cepas de Puebla y de la Ciudad de México pudieron haber adquirido una betalactamasa TEM-1 en eventos independientes. Sólo dos cepas portan la metalo-beta-

lactamasa NDM-1: la AN54 (de Puebla, México) en un plásmido y la sz1652 (de China) en el cromosoma, pero en ambos casos dentro del transposón característico en el que se suele encontrar. Si bien los macrólidos no se utilizan para tratar infecciones causadas por bacterias Gram negativas, la cepa sz1652 porta dos genes de resistencia a este tipo de antibióticos. Todo esto señala que A. haemolyticus ha estado adquiriendo genes de resistencia a antibióticos clínicamente relevantes, pues pueden complicar el tratamiento de las infecciones por esta bacteria y también pudieran diseminar los genes de resistencia entre otras bacterias.

La relación genotipo-fenotipo en los antibiogramas de las cepas mexicanas, que es de las que tenemos toda la información disponible, no es directa; es decir, que una cepa porte un gen de resistencia a antibiótico no garantiza que presente un fenotipo de resistencia, y a su vez, un fenotipo de resistencia no necesariamente se explica por la presencia de un gen de resistencia a antibióticos. Por ejemplo, dos cepas pueden tener los mismos genes de resistencia, pero una es sensible (AN7) y la otra es multi-drogo resistente (3281). Estas diferencias pueden deberse a que las secuencias de los genes de resistencia entre las cepas no son idénticas a nivel de nucleótidos, y pudiera haber desde diferencias en el nivel de expresión del RNA mensajero por el distinto uso de codones hasta diferencias a nivel de aminoácidos porque cambien los codones. Las diferencias a nivel de aminoácidos también pudieran impactar en la estructura tridimensional de la proteína o en su afinidad por el sustrato o su actividad catalítica. Asimismo, pudieran existir diferencias a nivel de promotores o reguladores transcripcionales. Otra posible fuente de distinción pudieran ser los distintos niveles de expresión y de actividad de las bombas de eflujo, ya que éstas confieren resistencia a una amplia variedad de antibióticos (no son específicas).

El genoma de *A. haemolyticus* tienen múltiples copias del gen que codifica para el factor de virulencia más nombrado en *A. baumannii*, OmpA, una proteína de superficie muy abundante, que es inmunogénica, puede inducir apoptosis en las células del hospedero, y que también se ha asociado con la formación del biofilm. Sin embargo, no en todos los genomas se encontraron todos los genes que codifican lo necesario para la síntesis de la cápsula, una envoltura que protege a la bacteria de la resequedad; pero es posible que falte caracterizar estos loci en *A. haemolyticus*, pues se sabe que en *A. baumannii* son altamente

variables. Por otro lado, todas las cepas tienen un gen que codifica para una fosfolipasa D y un sistema de secreción tipo 2 que la pudiera excretar para lisar las células del hospedero; además, 10 cepas contienen una fosfolipasa C adicional. Entonces, es posible que *A. haemolyticus* utilice estrategias similares a *A. baumannii* para causar daño a su hospedero y sobrevivir a la resequedad.

Nota: Los métodos y resultados detallados de mi investigación se encuentran descritos en el artículo "Chromosome Architecture and Gene Content of the Emergent Pathogen *Acinetobacter haemolyticus",* publicado en la revista *Frontiers in Microbiology* (Castro-Jaimes et al., 2020), que se encuentra a partir de la siguiente página.

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Chromosome Architecture and Gene Content of the Emergent Pathogen *Acinetobacter haemolyticus*

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Acinetobacter haemolyticus is a Gammaproteobacterium that has been involved in serious diseases frequently linked to the nosocomial environment. Most of the strains causing such infections are sensitive to a wide variety of antibiotics, but recent reports indicate that this pathogen is acquiring very efficiently carbapenem-resistance determinants like the *bla*NDM-1 gene, all over the world. With this work we contribute with a collection set of 31 newly sequenced nosocomial *A. haemolyticus* isolates. Genome analysis of these sequences and others collected from RefSeq indicates that their chromosomes are organized in 12 syntenic blocks that contain most of the core genome genes. These blocks are separated by hypervariable regions that are rich in unique gene families, but also have signals of horizontal gene transfer. Genes involved in virulence or encoding different secretion systems are located inside syntenic regions and have recombination signals. The relative order of the synthetic blocks along the *A. haemolyticus* chromosome can change, indicating that they have been subject to several kinds of inversions. Genomes of this microorganism show large differences in gene content even if they are in the same clade. Here we also show that *A. haemolyticus* has an open pan-genome.

Keywords: *Acinetobacter haemolyticus*, chromosome architecture, emerging pathogen, opportunistic pathogen, horizontal gene transfer, open pangenome

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INTRODUCTION

Species of *Acinetobacter* are widespread in nature. They can be isolated from different environments, such as soil, water, and food, and as commensals of many animals, including humans (Bouvet and Grimont, 1986; Doughari et al., 2011; Fyhrquist et al., 2014). Unfortunately, some *Acinetobacter* species are dangerous opportunistic pathogens of humans. The *Acinetobacter baumannii* – *Acinetobacter calcoaceticus* (ABC) complex is composed of closely related species that cause serious infections in the hospital setting and, less frequently, in the community (Gerner-Smidt and Tjernberg, 1993; Cosgaya et al., 2016; Nemec et al., 2015). The ability of *A. baumannii*, the most clinically relevant member of the ABC complex, to acquire antibiotic resistance genes has favored the appearance of multidrug-resistant (MDR) clones, and this characteristic combined

with its capacity to form biofilms and to survive desiccation allows the species to persist in the hospital environment and promote the emergence of outbreaks (Antunes et al., 2014; Doi et al., 2015). Infection by *A. baumannii* leads to worse clinical outcomes than those associated with other ABC complex species (Chuang et al., 2011; Fitzpatrick et al., 2015; Chen et al., 2018; Wisplinghoff et al., 2012; Lee et al., 2013; Park et al., 2013).

Acinetobacter haemolyticus belongs to the haemolytic clade; the members of this clade show beta-haemolysis halos in blood-agar media, and sometimes they can also degrade gelatin; *A. haemolyticus* shows both phenotypes (Bouvet and Grimont, 1986; Tayabali et al., 2012; Touchon et al., 2014; Nemec et al., 2016). The haemolytic clade has 13 named species (and 3 genospecies) to date, which have been isolated from humans, water and soil (Touchon et al., 2014; Nemec et al., 2016; Nemec et al., 2017, 2019), in contrast with six named species in the ACB clade, most of which are of clinical origin, but some have been isolated from soil (Nemec et al., 2011, 2019). *A. haemolyticus* has been implicated in serious infections, frequently in those linked to the nosocomial environment (Ko et al., 2007; Gundi et al., 2009; Turton et al., 2010; Fu et al., 2012; Schleicher et al., 2013; Wang et al., 2014; Jeong et al., 2016). Moreover, recent reports indicate that this pathogen is acquiring very efficient carbapenem-resistance determinants, such as the *bla*NDM-1 gene, worldwide (Fu et al., 2012; Jones et al., 2015; Bello-López et al., 2019; Jiang et al., 2019). All these characteristics suggest that *A. haemolyticus* may have the potential to become a threatening pathogen, following a path similar to that of *A. baumannii*.

In this work, we analyzed the genome sequences of a collection of 31 newly sequenced *A. haemolyticus* isolates obtained from different Mexican hospitals and a previously sequenced Mexican strain (Bello-López et al., 2019). Additionally, we added 12 complete genomes of *A. haemolyticus* isolates from other parts of the world from RefSeq. With all these data, we examined the genomic diversity of the collection and the evolutionary forces that have shaped the genome architecture of this species, with a particular focus on the roles of horizontal gene transfer (HGT) and gene gain and loss. This analysis contributes to our understanding of how the emergent pathogen *A. haemolyticus* evolves.

MATERIALS AND METHODS

The *A. haemolyticus* Collection

Mexican *A. haemolyticus* strains were obtained from different hospitals in different years, and only one sample per patient was considered, regardless of the antibiotic susceptibility profile. Considering that no previous data on the clonal relationship between strains and there were no studies on the diversity of Mexican strains circulating among hospitals, we kept all strains that fulfilled the above-mentioned criteria. Species identity was initially determined by querying the NCBI's nt database with the Sanger sequence of the cloned PCR product of a fragment of *rpo*B (Zone-1) with an identity cutoff of 97%; the results were the same if the cutoff was more strict (99%) (La Scola et al., 2006). Additionally, we downloaded all putative *A. haemolyticus* genomes available in the National Center for Biotechnology Information (NCBI) RefSeq until October 30th, 2018, including their associated clinical data.

Genome Sequencing and Assembly of Mexican *A. haemolyticus* Strains

Mexican isolates were sequenced with paired-end Illumina MiSeq 2 × 300 bp sequencing (except for strain 11616, which was sequenced with HiSeq 2 × 150 bp sequencing) by Macrogen, Korea, and Instituto Nacional de Medicina Genómica (INMEGEN), Mexico. Some genomes were sequenced with the PacBio RSII or PacBio Sequel platform at Yale and SNPsaurus; all reads are available in SRA under accessions SRR10672463–SRR10672503 (**Supplementary Table S1**).

Illumina reads were adapter filtered with Trimmomatic (Bolger et al., 2014) against a custom database with Illumina adapter sequences up to 2018 (document # 1000000002694 v04, January 2018). The resulting reads were quality trimmed with DynamicTrim, which is part of the SolexaQA suite (Cox et al., 2010); final quality was inspected with FastQC[1].

Filtered and trimmed Illumina reads were assembled using ABySS 2.0.1 (Simpson et al., 2009), SPAdes 3.9.0 (Bankevich et al., 2012), and Velvet 1.2.10 (Zerbino and Birney, 2008) with various kmers. The best assembly obtained with each program was selected for further use with Metassembler 1.5 (Wences and Schatz, 2015). Hybrid assembly was performed with SPAdes (Bankevich et al., 2012) and Unicycler (Wick et al., 2017). All assemblies were inspected for various metrics, such as N50, average contig length, and total assembly size with getAssemblyStats.py, available from GitHub[2].

Core Genome Phylogeny and Diversity Analyses

The Average Nucleotide Identity (ANI) of all the genomes in our collection was assessed with pyANI (Pritchard et al., 2016) with a relaxed cutoff of 93% (Rosselló-Móra and Amann, 2015) and a strict 96% cutoff (Richter and Rosselló-Móra, 2009) to determine which genomes were *A. haemolyticus*. We built a Maximum Likelihood (ML) phylogenetic tree with the core monocopy protein-coding genes from the complete dataset of each species with RAxML, excluding sequences with recombination signals (Stamatakis, 2014). We inspected the tree to select one or two genomes per clade. We further sequenced the selected Mexican strains with long reads to finish those genomes, and we kept the complete genomes available from NCBI. In this way, we focused the genome structure analyses only on complete chromosomes.

For Single Nucleotide Variant (SNV) analysis, we obtained the VCF file from core genome alignments with Parsnp and Gingr (Treangen et al., 2014) and converted it to hierBAPS format with PGDSpider (Lischer and Excoffier, 2012) and Perl. We ran Principal Component Analysis (PCA) with gdsfmt and SNPRelate (Zheng et al., 2017, 2012); Bayesian analysis was

[1]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

[2]https://github.com/semiramisCJ/spotsAhaem

performed with rhierbaps (Cheng et al., 2013), ape version 5.3 (Paradis and Schliep, 2019) and phytools version 0.6–99 (Revell, 2012), also in R 3.6.1 (R Core Team, 2019).

Genome Annotation

Initial genome annotation was performed with Prokka (Seemann, 2014), but the final annotations of the submitted genomes were performed by the NCBI staff with PGAP; all accession numbers are listed in **Supplementary Table S1**. Antibiotic resistance determinants were identified using the Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2016). Virulence factors were identified by using the Virulence Factor of Bacteria Database (VFDB) (Chen et al., 2016) and searching for secretion systems in the TXSSCAN profiles (Abby et al., 2014; Abby and Rocha, 2017). The capsule and outer core of lipooligosaccharide (LOS) loci from the genome sequences of *A. baumannii* strains A85 (KC118540.6), A91 (JN968483.3), D13 (HM590877.5), and SDF (BK010760.1) were analyzed separately. Iron-acquisition systems were curated from the literature (Dorsey, 2003; Zimbler et al., 2009; Antunes et al., 2011; Eijkelkamp et al., 2011; Hasan et al., 2015; Penwell et al., 2015) and cross-referred with the genomes of *A. baumannii* strains ACICU (NC_010611.1), AYE (CU459141.1), and 8399 (AY149472.1). Clusters of Orthologous Groups (COG) and Non-supervised orthologous group (NOG) functional annotation was performed with the eggNOG mapper (Huerta-Cepas et al., 2017, 2016).

To identify mobile genetic elements in the representative genomes, we used other specialized databases and tools: we queried the Mobile Genetic Elements Database (MGE DB) (Pärnänen et al., 2018) and, to identify integrative and conjugative elements (ICEs), ICEberg database version 2.0 (Liu et al., 2019) with the local version of ICEfinder (Hong Yu), which requires the EMBOSS suite (Rice et al., 2000). We searched for insertion sequences (ISs) with ISEScan 1.6 (Xie and Tang, 2017) and ISFinder (Siguier et al., 2006). Phages were analyzed with PHASTER (Arndt et al., 2016) and VirSorter (Roux et al., 2015). To detect signals of HGT via the nucleotide composition of each genome, we used AlienHunter (Vernikos and Parkhill 2006) and a custom python script to calculate the GC content for each replicon.

Syntenic Block (Spot) Delimitation

The code used for spot delimitation is available from GitHub see text footnote 2. We based our analysis on the methodology proposed by Oliveira et al. (2017), with the following modifications: first, we separated protein-coding genes into orthologous groups with PanOCT (Fouts et al., 2012). Next, we focused on single-copy core orthologs, and if the vicinity of a defined gene was shared by all the strains, the genes were said to be in an interval. This analysis was performed for overlapping sliding windows with a step of one gene. The vicinity was set to five genes upstream and five genes downstream of the middle (query) gene (a total of 11 genes at a time), and the vicinity had to share at least three other genes (a total of at least four genes in common), no matter the order; moreover, the genes at the extremes of the interval had to belong to the same gene family, allowing for permutations (**Figure 5**).

Overlapping intervals of each strain were combined into superintervals to avoid redundancy. To map equivalent superintervals between strains, we used the intersection of gene families and kept a link table; in our analysis, all the mappings resulted in 1-to-1 agreement, with each superinterval mapping to one superinterval and no splits.

Finally, we added the non-core genes to the superintervals to obtain the complete syntenic blocks (spots), and the genes outside spots were said to be in hypervariable regions. Spots were consecutively named as encountered by the script. If the spots shared the identifier, they were said to be equivalent. Hypervariable regions were named on the basis of the surrounding spots to keep track of their genomic context in each strain.

Recombination Signal Detection

To detect gene families with recombination signals, we first aligned the proteins with Clustal Omega (Sievers et al., 2014) and used RevTrans 1.4 (Wernersson and Pedersen, 2003) to guide nucleotide alignment and keep the alignments in frame. Then, we used Phi-pack (Bruen et al., 2006) to test for recombination; if the *p*-value of the phi test with permutation was less than 0.05, we considered the alignment to have signals of recombination, as in Wang et al. (2016).

Context and Comparison of Common Gene Families

To determine how many times each gene family was represented in each spot or hypervariable region and how similar the genetic compositions of the family members were, we searched each gene family, accounting for paralogs, among all locations between all strains. The Jaccard index was computed as the overlap (intersection) between sets; thus, a value of 1 meant complete overlap, and 0, complete dissimilarity. Jaccard indexes were computed among equivalent spots and among hypervariable regions with the same genetic context (flanked by the same spots).

Category Enrichment

The enrichment of some categories was assessed by hypergeometric tests, corrected for multiple testing, in R 3.6.1 (R Core Team, 2019). For each category (spot or hypervariable region), we searched for COG/NOG functional enrichment, virulence factors, phages, genes with a recombination signal, and atypical nucleotide composition, the last of which was determined by both AlienHunter and GC content.

Data Visualization

The ML phylogenetic tree was annotated with iTOL (Letunic and Bork, 2019). Plots of genome features per genomic position were constructed with matplotlib (Hunter, 2007) in Python 3. The rest of the plots were constructed in R 3.6.1 (R Core Team, 2019). All heatmaps were created with ComplexHeatmap (Gu et al., 2016); scatterplots and violin plots were created with ggplot2 (Wickham, 2009). Additional R packages used included

dplyr, ggrepel, GGally, paletteer and RColorBrewer (Neuwirth, 2014; Schloerke et al., 2018; Hvitfeldt, 2019; Slowikowski, 2019; Wickham et al., 2019).

RESULTS AND DISCUSSION

Genome Collection

To study the genome architecture of the emerging pathogen *A. haemolyticus*, we constructed a data set consisting of the genome sequences of 31 Mexican isolates described here and a previously sequenced Mexican *A. haemolyticus* (Bello-López et al., 2019) (in total, 9 Mexican strains now have finished chromosomes) and 19 putative *A. haemolyticus* complete genome sequences available in NCBI RefSeq database (4 of them were finished assemblies), which were isolated from hospitals in different countries. To confirm the species designation of all the genomes in the collection, and to detect equivalent strains, we calculated the average nucleotide identity (ANI) between all genome pairs. Our results confirmed that all but two isolates were correctly assigned to *A. haemolyticus* (**Supplementary Figure S1** and **Supplementary Table S2**). The two exceptions were isolates JKSF06 and KCRI-45, which were excluded from the final data set because they had around 84% ANI values (**Supplementary Figure S1** and **Supplementary Table S2**), far below the species designation cutoffs of 93–96% (Richter and Rosselló-Móra, 2009; Rosselló-Móra and Amann, 2015). We excluded strain TG19602 because its genome assembly was very fragmented and could introduce noise in the genome content analysis: it had 382 contigs (the largest of all the collection) and the lowest average contig length. To avoid redundancy and to work only with good quality data, we only kept the assembly with the best quality when there were equivalent strains; in those cases, the ANI values were of 99.9% (**Supplementary Table S2**). Thus, CIP 64.3 (the type strain of the species) represented assemblies of MTCC 9819, NBRC 109758, and TG19599, whereas the assembly of ATCC 27244 represented the TG21157 genome (Marcus et al., 1969; Khatri et al., 2014; Touchon et al., 2014).). After applying all these filters, our final data set consisted of 44 genomes. Their corresponding assembly status (finished or draft), isolation year, isolation country, and accession numbers are listed in **Table 1**.

Mexican *A. haemolyticus* strains were isolated from different patients, sources, hospitals, and hospital units in different years, irrespective of antibiogram results. Most of the isolates were collected from secondary and tertiary care institutions located in Puebla and Mexico City. Most of the patients were admitted to the Internal Medicine Unit (10), Oncology (7) or Emergencies (6), but some patients were also in Surgery (2) or Intensive Therapy (2), and 1 patient was HIV+. The average and median ages of hosts were 25 and 15.5 years, respectively, because more than half of the samples (18/32) were from pediatric hospitals. Twelve of the patients were female. Isolation year ranged from 1998 to 2016. The most common isolation sources were peritoneal dialysis fluid (14), blood (6), and bronchial secretion (4).

Many of the NCBI genome sequences were from China (4). In addition, one of the sequences was from the Czechia, one was from Tanzania, and one was from the United States; the remaining strains (5) did not have information about the country from which they were isolated. The most common isolation source was sputum (6). Our collection also included an isolate (HW-2A) obtained from an E-waste recycling plant, in contrast to the rest of the strains, which had a clinical origin and isolation dates ranging from 1962 to 2017.

General Features of the Genomes in the Collection

Draft genomes of all Mexican isolates were obtained with an Illumina platform. Additionally, the genome sequences of eight of them were completed with the aid of PacBio reads (see section "Materials and Methods"; **Supplementary Table S1**). This genome collection represents the largest data set of sequenced clinical *A. haemolyticus* isolates to date and an important step in our understanding of an emergent pathogen that has received little attention due to misidentification with routine techniques and because most of its isolates tend to be antibiotic sensitive.

Genome size was similar among all the strains, with a median of 3.5 Mb. The assembly size of Mexican *A. haemolyticus* genomes in the collection ranged from 3 264 943 to 3 694 983 bp (*sd* = 117 494.84 bp), whereas that of NCBI genomes ranged from 3 291 819 to 3 715 198 bp (*sd* = 127 635 bp). Among the complete genomes, chromosome size ranged from 3.3 to 3.7 Mb (*sd* = 1.1 Mb), and some strains had up to six plasmids (mean = 3). Plasmid size ranged from 4 280 to 107 843 bp (*sd* = 3 0532.56 bp).

The *A. haemolyticus* genomes analyzed here contained 3 028 to 3 720 protein-coding genes (*sd* = 162) and could be organized into 10 866 gene families, only 1 893 of which formed the core genome and 4 941 were singletons (**Supplementary Figure S2**). We also performed a sampling analysis in which we monitored the increase in pangenome size when more genomes were included (**Supplementary Figure S3**). These data indicated that *A. haemolyticus* has an open pangenome.

Diversity, Distribution, and Grouping of the Strains

To evaluate the relationships between the strains, we analyzed the SNVs in the core genome (regardless of whether they were in non-coding or coding regions), and we built a ML phylogenetic tree with all monocopy core protein-coding genes without recombination signals. The number of SNVs in the complete core genome between most pairs of strains were in the order of thousands, which highlights the diversity of the dataset. The exceptions were a few cases of closely related strains: 3 SNVs between AN43 and AN59 even if they were from the same hospital, but isolated from different patients at different years; 6 SNVs between CIP 64.3 and strains NCTC 10305 and NCTC 12155, which are also very similar at the ANI values; 10 SNVs between AN4 and 10633 even if they were isolated from different hospitals, in different parts of Mexico, at different years (**Table 1** and **Supplementary Table S3**). In the ML phylogenetic tree (**Figure 1** and **Supplementary Figure S4**), the Mexican and Chinese strains were interspersed. The isolates from the same Mexican hospital belonged to different clades,

TABLE 1 | Assembly status, isolation country, isolation year, and assembly accession numbers of *A. haemolyticus* genomes analyzed in this work.

Notes	Strain	Country	Year	Assembly accession numbers	References
Mexican strains, finished					
	INNSZ174	Mexico	1998	CP031998 - CP032001	This study
	11616	Mexico	2012	CP032002 - CP032008	This study
	AN43	Mexico	2015	CP031976 - CP031978	This study
	AN54	Mexico	2016	CP041224 - CP041229	Bello-López et al., 2019
	AN59	Mexico	2016	CP031972 - CP031975	This study
	2126ch	Mexico	2011	CP031991 – CP031997	This study
	5227	Mexico	2014	CP031988 – CP031990	This study
	AN3	Mexico	2010	CP031984 – CP031987	This study
	AN4	Mexico	2010	CP031979 – CP031983	This study
NCBI's strains, finished					
	TJS01	China	2012	NZ_CP018871 – NZ_CP018873	
	XH900	China	2016	NZ_CP018260 – NZ_CP018261	
	HW-2A	China	2017	NZ_CP030880	
	sz1652	China	2017	CP032135 – CP032137	Jiang et al., 2019
Mexican strains, draft					
	2227	Mexico	2011	WTTY00000000	This study
	3275	Mexico	2012	WTTX00000000	This study
	3281	Mexico	2012	WTTW00000000	This study
	5439	Mexico	2014	WTTV00000000	This study
	10633	Mexico	2013	WTTU00000000	This study
	11650	Mexico	2013	WTTT00000000	This study
	11652	Mexico	2010	WTTS00000000	This study
	11654	Mexico	2008	WTTR00000000	This study
	11658	Mexico	2008	WTTQ00000000	This study
	978H	Mexico	2016	WTTP00000000	This study
	AN10	Mexico	2012	WTTO00000000	This study
	AN11	Mexico	2012	WTTN00000000	This study
	AN13	Mexico	2012	WTTM00000000	This study
	AN20	Mexico	2013	WTTL00000000	This study
	AN27	Mexico	2014	WTTK00000000	This study
	AN34	Mexico	2014	WTTJ00000000	This study
	AN44	Mexico	2015	WTTI00000000	This study
	AN5	Mexico	2011	WTTH00000000	This study
	AN60	Mexico	2016	WTTG00000000	This study
	AN61	Mexico	2016	WTTF00000000	This study
	AN63	Mexico	2016	WTTE00000000	This study
	AN7	Mexico	2011	WTTD00000000	This study
	DIV33	Mexico	2016	WTTC00000000	This study
NCBI's strains, draft					
	ATCC 19194			NZ_GG770435.1 – NZ_GG770495.1	Baumann et al., 1968
	ATCC 27244	United States		NZ_GG665949.1 – NZ_GG666013.1	Marcus et al., 1969
	CIP 64.3 T			NZ_KB849798.1 – NZ_KB849812.1	Baumann et al., 1968; Touchon et al., 2014
	NIPH 261	Czechia	1993	NZ_KB849813.1 – NZ_KB849819.1	Nemec et al., 2000; Touchon et al., 2014
	KCRI-348C	Tanzania	2014	NZ_OVCN01000001.1 – NZ_OVCN01000043.1	
	NCTC 10305		1962	NZ_UFRR01000001.1 – NZ_UFRR01000006.1	
	NCTC 10306		1962	NZ_UFRT01000001.1 – NZ_UFRT01000004.1	
	NCTC 12155		1987	NZ_UAPN01000001.1 – NZ_UAPN01000032.1	

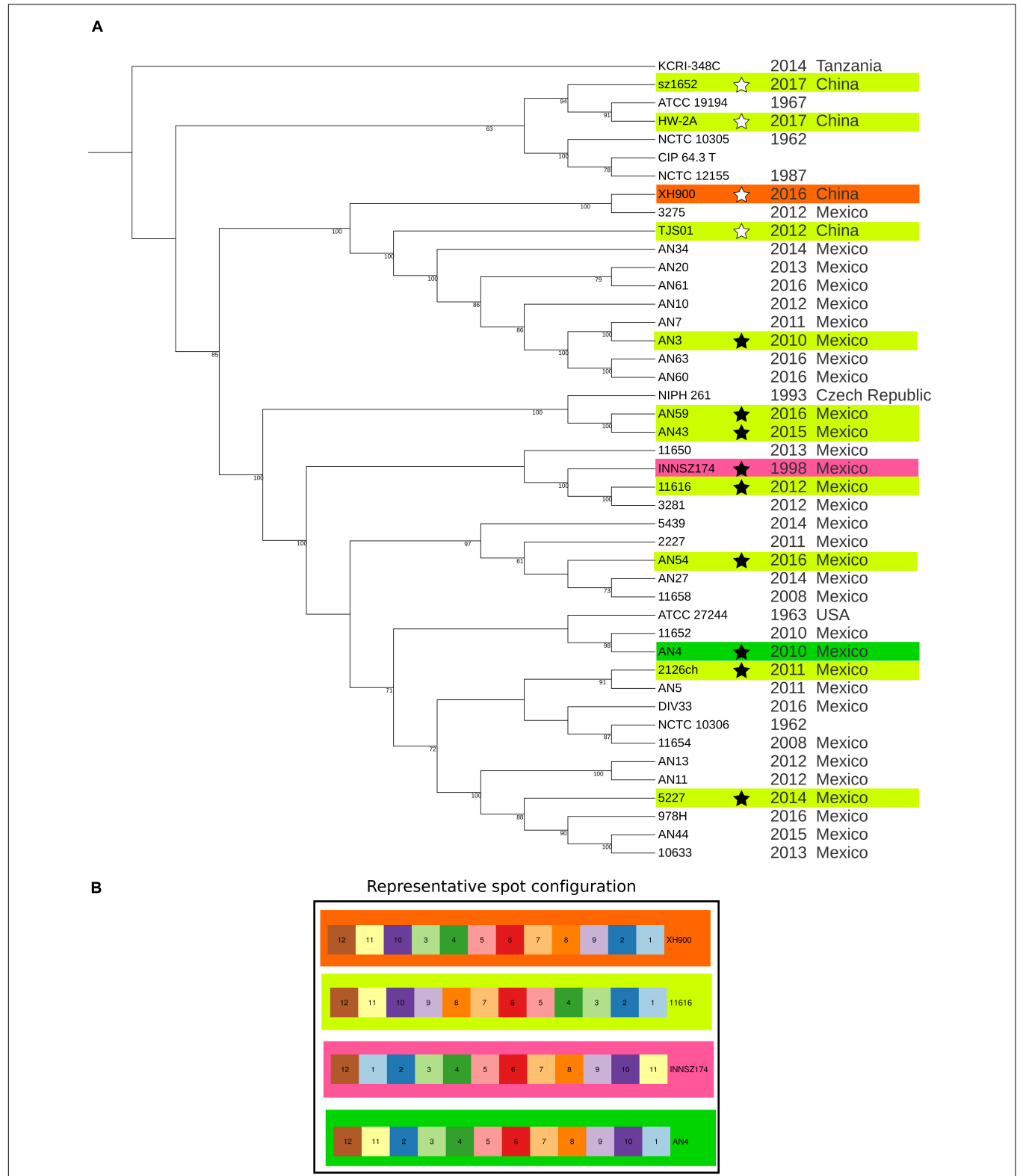

FIGURE 1 | Maximum likelihood phylogeny of core protein-coding genes and spot configuration in representative genomes. **(A)** Maximum likelihood phylogeny. The ML tree was built with the 955 core monocopy orthologous protein-coding genes without recombination signals between the 44 analyzed A. haemolyticus genomes. The tree was drawn with iTOL. Annotation includes isolation year and isolation country. Stars mark representative genomes. Branch length is not to scale. Bootstrap values above 60% are shown. **(B)** Graphic representation of spot configuration of representative genomes. The background color is used to clarify where the spot configuration is located in the representative genomes in the phylogeny.

and strains obtained in the same year were located in different positions on the tree (**Figure 1**). We also found that there were multiple, distantly related lineages circulating in Chinese hospitals. These data showed that *A. haemolyticus* clones were introduced to Mexican and Chinese hospitals during multiple independent events.

We analyzed the SNVs present in the complete core genome with two approaches, PCA and a Bayesian method (rhierbaps). The results of the two strategies were consistent, but the Bayesian method tended to split PCA clusters into many subpopulations. The results of both SNV analyses were consistent with the bipartitions of the ML tree because they showed the same patterns (**Supplementary Figure S5**): some strains formed clusters clearly separated from others by isolation year or isolation country. A few strains were very different from the rest of the strains in the collection, and the others did not form sharply delimited clusters. The first scenario was illustrated by two clusters: some strains isolated in 1962 (NCTC 10305 and NCTC 12155) formed a tight group with the type strain of *A. haemolyticus* (CIP 64.3), also isolated in the 1960s (Baumann et al., 1968). Most of the strains isolated in Puebla (Mexico) from 2011 to 2016 (AN3, AN7, AN10, AN20, AN34, AN60, AN61, and AN63) were more similar to each other than to members of the other clusters. The rest of the Mexican *A. haemolyticus* strains grouped more tightly with each other than with the strains from other countries. The most heterogeneous strains were those isolated in Tanzania (KCRI-348C) and China (HW-2A, XH900, sz1652 and TJS01) from 2012 to 2017. This analysis showed that most *A. haemolyticus* genomes were grouped by isolation country; nonetheless, we identified a cluster of closely related strains grouped by isolation year.

Genome Architecture of Representative Strains

We randomly selected at least one genome per clade and defined it as the representative genome of each clade. We excluded two clades (CIP 64.3 and KCRI-348C) from the genome architecture analyses because none of the chromosome sequences of their members were finished. To evaluate the differences in genome architecture between members of our *A. haemolyticus* collection, we first identified the syntenic regions in all of them using, with some modifications, a previously suggested method based on the proximity of orthologous protein-coding genes (Oliveira et al., 2017). Briefly, we identified all the orthologous genes among all the genomes and focused on monocopy core orthologous genes to determine if they were present within the same region in a defined window in all genomes. At this point, we omitted all multicopy and accessory genes. A set of core genes with a conserved position formed an "interval." Then, we reincorporated multicopy and accessory genes into the intervals to generate "spots." The zones between spots were named "hypervariable regions." The hypervariable regions were composed of genes that did not pass the synteny criteria. Importantly, we limited our analysis to protein-coding genes and excluded pseudogenes. This method identifies syntenic regions based only on the conservation of core orthologous genes, regardless of the accessory genome content, and is flexible because the accessory genes do not obscure the conserved regions.

With the implemented method, we identified 12 spots (syntenic regions) and 7 to 9 hypervariable regions (zones in the genome flanked by spots) in the *A. haemolyticus* genomes, as in some strains, a few pairs of spots were not separated by hypervariable regions. The spots were always larger than the hypervariable regions. Most (2 608 to 3 038 genes; *sd* = 120) of the genes were located within spots comprising 93 to 97% of the genes on the chromosome. Each spot contained 43 to 555 genes (*sd* = 150). On the other hand, hypervariable regions contained, in total, between 85 and 221 genes (*sd* = 42) per chromosome, and each hypervariable region included 1 to 102 genes (*sd* = 24) (**Supplementary Figure S6**).

The relative order of the spots tended to be conserved. However, we observed various inversions that always involved spots 1, 2, 10, and 11, which led to four different spot configurations (**Supplementary Figure S7** and **Figure 1**); in contrast, spot 12 always had the same relative orientation in all the chromosomes. The most frequent spot configuration, represented by strain 11616, was present in multiple clades, and the other three configurations detected in distinct clades indicated that rearrangements were possible but infrequent.

Gene Order Inside Spots

To analyze the order of core genes inside equivalent spots, we selected a genome from each of the four spot configurations described above as references, namely, INNSZ174, AN4, XH900 and a strain with the most common configuration (11616).

Almost all the strains with the same spot configuration as 11616 had the exact same order of core genes; strains AN3 and TJS01 were the exceptions because they had a complete inversion of spot 6 (**Figure 1**). When we compared strain 11616 with strain INNSZ174, which belonged to the same clade but had a different spot configuration, we found that the *relative* core gene order in all spots was conserved (**Figure 1**). When we compared strain 11616 with strain AN4, which belonged to a neighboring clade, the inversions inside the spots reflected the inversions between the spots, as the only conserved core gene positions were those of spots 1, 11, and 12, which had the same order (**Figure 1**). Finally, when we compared strain 11616 with strain XH900, which belonged to a more distant clade, we saw that again, the spots with the same order also had conserved core gene positions inside them; this was the case for spots 1, 2, 6, 10, 11, and 12, but the rest had complete inversions (**Figure 1**). All these data showed that the relative order inside spots was conserved and that the most common rearrangements (inversions) involved multiple spots.

Gene Content in Spots and Hypervariable Regions

To evaluate how similar the spots were in terms of gene content and to determine how many genes in the hypervariable regions were shared among strains, we computed the Jaccard index, which indicates how similar two datasets are, with 1 indicating identical and 0 indicating completely dissimilar.

Two equivalent spots contain the same core genes; however, they can differ in the type and number of accessory genes. To assess how similar the spots were, we compared their total gene contents. All spots shared at least 50% of their genes, but others were nearly 100% percent identical (**Figure 2**). At least 50% of the shared genes comprised the monocopy core orthologous genes that were used to identify the spots.

We also found that some spots were more similar in global gene content than others, irrespective of size. For example, spots 2 and 12 (median sizes of 96 and 47 genes, respectively) were the most conserved spots in all the strains, and spots 6 and 7 (median sizes of 369 and 135 genes, respectively) were the most conserved in more closely related clades. The most heterogeneous spots were spots 3, 5, and 8 (median gene contents of 63, 507, and 288, respectively).

These findings indicated that accessory genes are the drivers of diversity in gene content among spots and that even if two equivalent spots have similar sizes, their gene contents can be very different.

Next, we analyzed the gene content of hypervariable regions flanked by the same spots, i.e., with the same genetic context, in different strains. We found a range of patterns (**Figure 3**): at one extreme, there were no regions separating spots, and in other cases, there were similar gene contents within equivalent hypervariable regions; at the other extreme, there were equivalent hypervariable regions that were completely different in terms of gene content, or there were unique hypervariable regions present in only one or two strains.

The first scenario occurred when a pair of spots were always together but without hypervariable regions in between. Spots 9 and 10 exemplified this case. These spots formed separate entities because they were in different configurations in some strains (**Supplementary Figure S7** and **Figure 1**), considering that they could not form a larger spot overall and thus did not comply with the synteny criteria.

Five hypervariable regions with the same genetic context also possessed equivalent genes. Some of them shared only one or two genes, as in the case of the hypervariable region between spots 1 and 12; meanwhile, others shared a larger gene set among all or a subgroup of strains. The latter case sometimes also occurred when a gene context could not be present in other strains because the involved spots were not near each other; for example, the hypervariable region between spots 2 and 3 was present in most of the genomes but absent in strain sz1652, whereas these spots were separated in strain XH900 because they were not consecutive in its genome.

We identified four hypervariable regions with the same genetic context but with very different gene contents. For example, the hypervariable region between spots 1 and 2 was very heterogeneous: in some of the genomes, it was composed of 1 to 8 genes, but in strain AN54, it contained 58 genes, many of which were related to transposases associated with ISs. Furthermore, this region was absent in strain HW-2A (see **Supplementary Figure S8**).

We identified two hypervariable regions that were unique on the basis of either gene content or genetic context. One was the hypervariable region between spots 5 and 6; it included two genes (a hypothetical protein and a transposase) and was present only in strains AN3 and TJS01. These two regions shared a hypothetical protein, but they had different transposases. The other case was a hypervariable region between spots 2 and 11 in strain AN4; this arrangement was unique because only this strain exhibited these spots as adjacent fragments. The region consisted of a gene coding for a transposase and another coding for a hypothetical protein; the latter was also found in the hypervariable region between spots 1 and 2 in seven strains (2126ch, AN3, AN43, AN59, INNSZ174, sz1652, and TJS01).

All these results highlighted that hypervariable regions, which are located along the entire chromosome, are the most susceptible regions to gene gain and loss. Hypervariable regions can be highly heterogeneous in both gene content and size. These regions might be hotspots for gene content variation, in some cases, due to site-specific recombination driven by transposases.

Functional Categories in Spots and Hypervariable Regions

To obtain a general overview of the functions of the protein-coding genes within spots and hypervariable regions, we performed enrichment analysis of general functional annotations with COG and NOG categories. We found that two general metabolic categories were overrepresented among spots: "(C) Energy production and conversion" and "(J) Translation, ribosomal structure and biogenesis." In contrast, in hypervariable regions, there were both an excess of genes without functional annotation and enrichment in the category "(L) Replication, recombination and repair," which is frequently linked to regions with HGT signals.

Mobile Genetic Elements and Other Horizontal Gene Transfer Signals

Horizontal gene transfer is an important contributor of genes associated with phenotypes of clinical concern in gram-negative bacteria that cause opportunistic infections, such as antibiotic resistance genes and virulence factors (Dobrindt et al., 2004). Foreign regions such as genomic islands have an atypical nucleotide composition, a skewed GC content, and one or more hallmarks of mobility, such as ISs, transposons, integrase attachment sites, integrases and even conjugation machinery (Dobrindt et al., 2004). Moreover, finding a specific genome segment within other genomic contexts in different strains or even species provides additional and strong evidence that it has a foreign origin (Ploswiki et al., 2015). Thus, to identify mobile genetic elements or chromosomal regions with HGT signals in representative genomes, we followed three steps. First, we used specialized software that may suggest HGT events of genomic regions, such as recombination signals (pairwise homoplasy index, phi), GC content and nucleotide composition (AlienHunter). In addition, we quantified the number of unique gene families, i.e., those present in only one strain. Then, we built non-redundant databases of HGT regions to obtain only a representative of each genomic fragment and then performed BLASTn searches against the non-redundant nucleotide database, excluding either the species *A. haemolyticus*

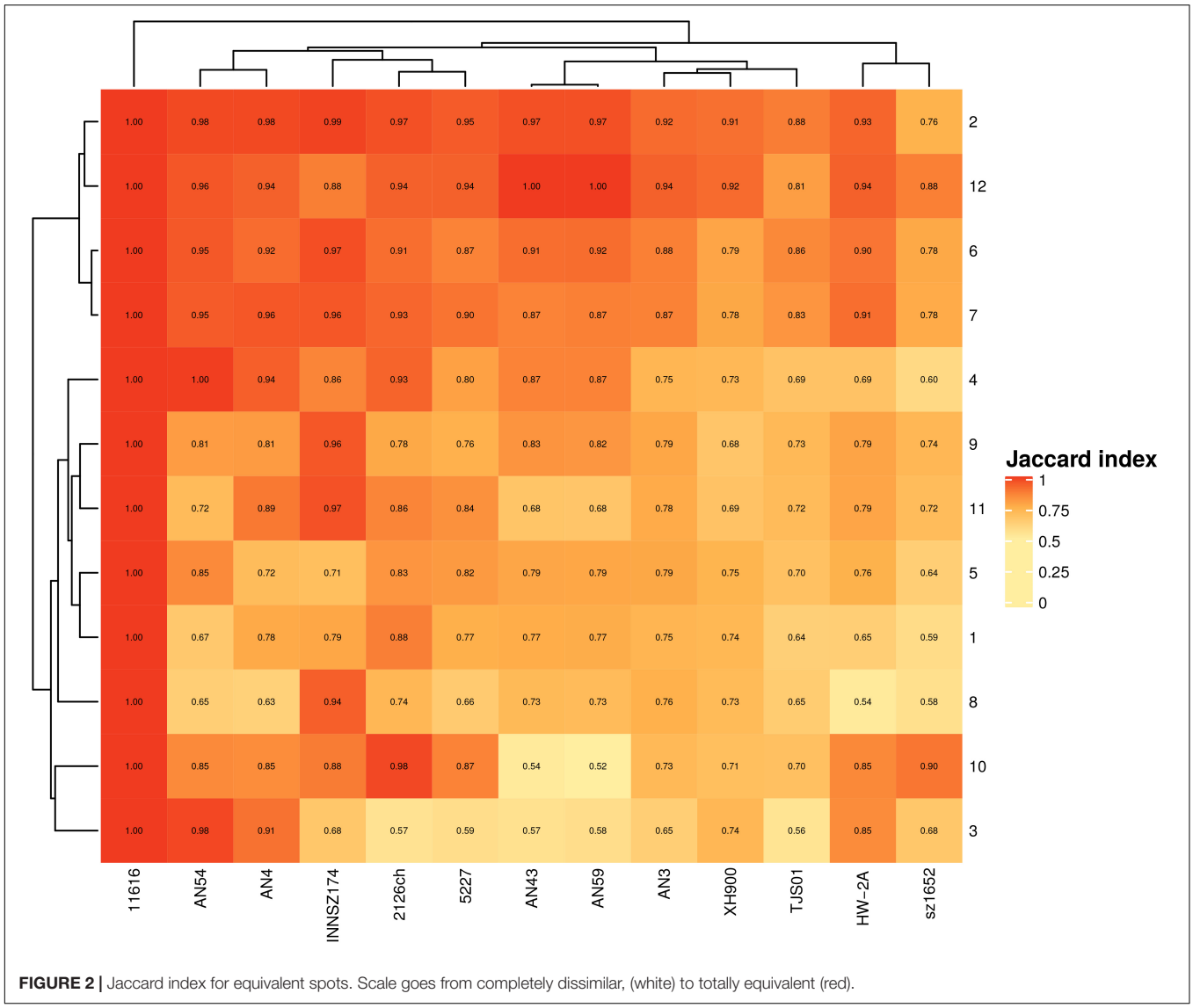

FIGURE 2 | Jaccard index for equivalent spots. Scale goes from completely dissimilar, (white) to totally equivalent (red).

or the genus *Acinetobacter*. Furthermore, we consulted databases of mobile genetic elements such as ISs, phages, and ICEs.

We found 695 to 797 (*sd* = 32) genes with recombination signals (detected by the phi test) per chromosome. Larger spots tended to have more genes with recombination signals (**Supplementary Figure S9**). Genes with recombination signals were overrepresented in spots of strains 11616, 2126ch, 5227, AN 3, AN 54, and sz1652. This observation showed that the presence of genes with recombination signals was related to spot size, such that the spots grew via the integration of genes by homologous recombination and gene duplication.

We also quantified gene families that were present in only one strain; these were considered unique gene families and were present in either one or multiple copies in the same genome. We found 29 to 374 unique gene families per chromosome, and they were present in both spots and hypervariable regions. We discovered regions without unique gene families but also hypervariable regions composed almost entirely of unique gene families (**Supplementary Figure S10**). Indeed, unique gene families were overrepresented in hypervariable regions in almost all representative strains (8/13). This finding showed that there is extensive gene content variation in *A. haemolyticus* chromosomes, mainly in hypervariable regions.

AlienHunter detected, in the chromosomes, putative HGT zones that varied from 5 to 15 Kb in size and overlapped with regions of atypical GC content (**Supplementary Figure S8**). These regions were almost always overrepresented in hypervariable regions compared with spots. The exception was strain HW-2A (with no enrichment), possibly because this strain also contained a high density of putative HGT regions along all regions of the chromosome; therefore, no enrichment was detected in any zone. We found that only a few of these regions were outside the genus *Acinetobacter*. One of these regions was the transposon that harbors the NDM-1 Metallo-beta-lactamase gene, which is present in two *A. haemolyticus* strains, namely, AN54 (plasmid) and sz1659 (chromosome), and found in a

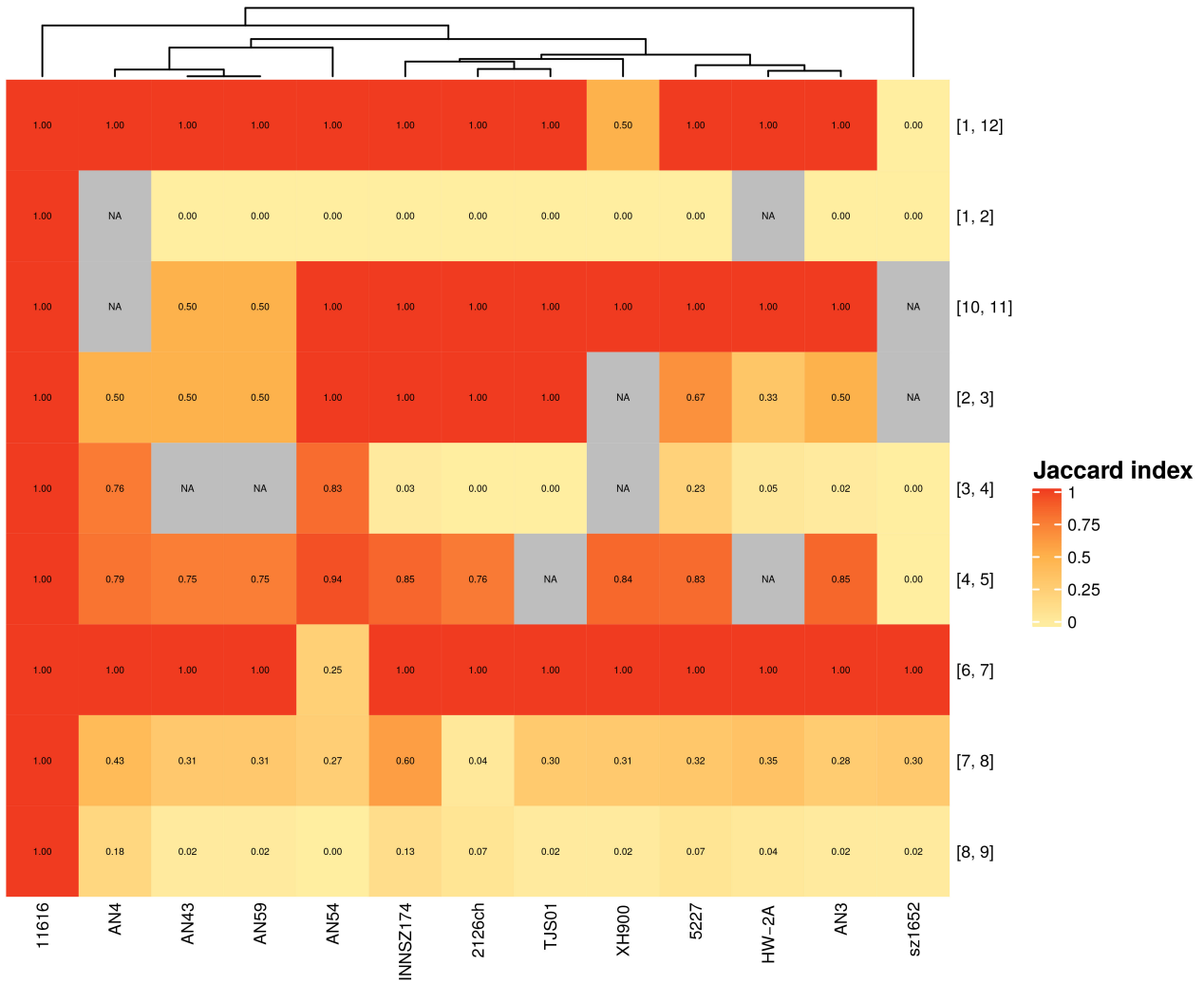

FIGURE 3 | Jaccard index for hypervariable regions between equivalent spots. Scale goes from completely dissimilar, (white) to totally equivalent (red); NA (gray)- Not applicable. Numbers in brackets represent the spots flanking each hypervariable region.

variety of bacterial species. The other region was present in strain 11616 and matched a chromosomal region of an uncharacterized gamma-proteobacterium isolated from the bee gut. Regarding the genus *Acinetobacter*, the putative HGT regions identified with AlienHunter were mainly found in *Acinetobacter junii* and *A. baumannii* plasmids and comprised hypothetical proteins, oxidoreductases, and transposases. These observations suggested that *Acinetobacter* species are donors of foreign genetic material with potential clinical relevance, such as the NDM-1 transposon, to *A. haemolyticus*.

Integrative and Conjugative Elements

Integrative and conjugative elements encode transposases but can also contain a variety of genes, and they are flanked by repeats (*att* sites) necessary for site-specific recombination. ICEs are inserted into the bacterial genome and encode all the elements required for their transfer by conjugation (Johnson and Grossman, 2015). In contrast to ICEs, integrative and mobile elements (IMEs) do not have complete conjugation machinery but can be transferred if the lacking mobile elements are provided by another ICE or plasmid (Delavat et al., 2017). We found some IMEs, which ranged in size from 3 688 to 79 964 bp (*sd* = 23171) and differed in both sequence and gene annotation, except in strains AN43 and AN59, which shared the same IME on the same chromosome. Additionally, none of these regions were found outside the *Acinetobacter* genus.

We found one to four putative IMEs in the chromosomes of nine strains: 11616 (1), 2126ch (1), 5227 (1), AN43 (1), AN59 (1), INNSZ174 (4), sz1652 (1), TJS01 (2), and XH900 (1). Chromosomal IMEs were located in spots 3 (INNSZ174), 5 (AN43, AN59, INNSZ174, and TJS01), 8 (11616, 2126ch, 5227, INNSZ174, sz1652, and TJS01), and 11 (XH900) or in a hypervariable region (between spots 3 and 4 in INNSZ174) (**Supplementary Figure S8**). In addition, ICEfinder detected 1 putative IME, without its characteristic flanking direct repeats, in the plasmids of two strains (plasmid unnamed2 of sz1652 and

pAHTJS2 of TJS01), which seemed to be conjugative plasmids. The conjugation machinery encoded in plasmid unnamed2 was very similar to that found in *A. baumannii* plasmids such as pACICU2 and pAba3207b, suggesting that *A. haemolyticus* can acquire and maintain plasmids present in *A. baumannii*.

Phages

PHASTER found 1 to 8 (*sd* = 2) putative phages in all strains. In contrast, VirSorter predicted only the largest plasmid (80 Kb) of strain 11616 (pAhae11616_f) as a putative phage region, probably because the plasmid is abundant in transposases; thus, we discarded VirSorter results in downstream analyses. Putative phage regions also overlapped with atypical GC content, which supported the foreign origin of these genomic fragments.

Transposases and Insertion Sequences

The mobile genetic elements database (MGE DB) identified only a transposase, tnpA-like, in each genome. However, there were 7 to 107 (*sd* = 33) genes annotated as transposases per chromosome. Therefore, we did not consider these results in further analyses.

Insertion sequences are DNA sequences composed of flanking sequences for site-specific recombination and a gene that codes for a transposase, which can be used to classify these elements into different families (Patricia Siguier et al., 2015). ISs are widely distributed in *A. haemolyticus* genomes; we identified 10 to 202 elements (*sd* = 61) per chromosome. The most abundant families were IS66, IS1, IS701, IS30, IS4, IS5, and IS3 because they were present in multiple copies, ranging from 2 to 13 copies (*sd* = 5) for IS66 and from 5 to 71 copies (*sd* = 24) for IS3. IS66 is a promiscuous IS with no sequence specificity (Patricia Siguier et al., 2015); this might explain why it is so frequent in the *A. haemolyticus* chromosome. IS66 has been found to interrupt a competence gene (*comEC*) in *A. baumannii* isolated in Italy (Gaiarsa et al., 2019). IS1 has been found in an *A. baumannii* transposon that harbors a gene that codes for a chloramphenicol acetyl-transferase (Elisha and Steyn, 1991); it is also present in Enterobacteriaceae plasmids (Nyman et al., 1981) and chromosomes (Lee et al., 2016), where it can mediate, in combination with IS3, IS4 and IS5, large insertions and deletions, some of the latter of which are mediated by recombination between adjacent ISs of the same family.

ISAba11, a member of the IS701 family (Rieck et al., 2012), in *A. baumannii* has been implicated in colistin resistance achieved by disruption of either of two genes important for lipid A biosynthesis (Moffatt et al., 2011). In addition, ISAba11 mediates the transposition of a genomic island that confers sulfonamide resistance from a plasmid to the *A. baumannii* chromosome (Hamidian and Hall, 2017).

IS18, a member of the IS30 family, activates an aminoglycoside resistance gene in *A. baumannii* by providing a functional promoter (Rudant et al., 1998). In a similar way, ISAba1, a member of the IS4 family, when located upstream, can potentiate the expression of intrinsic beta-lactamases, thus conferring an antibiotic resistance phenotype; for example, when ISAba1 is inserted upstream of $bla_{\mathrm{OXA-23}}$, the strain can be resistant to carbapenems (Nigro and Hall, 2016), and when ISAba1 is located upstream of *ampC*, the strain can be resistant to cephalosporins (Hamidian and Hall, 2014). In contrast, disruption of the *ampC* gene by insertion of IS5 (Said et al., 2018) or disruption of $bla_{\mathrm{OXA-75}}$ by IS3 (Li et al., 2015) results in strains susceptible to cephalosporin and, in the absence of other carbapenemases, the generation of carbapenem-susceptible isolates, respectively.

In summary, regions with atypical nucleotide features and unique gene families are overrepresented in hypervariable regions, whereas genes with recombination signals are more common in spots. IMEs are commonly found in spots, whereas ISs are scattered along the entire chromosome, are present in multiple copies, and can provide the substrate for genome rearrangements and modify distinct phenotypes.

Antibiotic Resistance Determinants in All *A. haemolyticus* Strains

We searched for antibiotic resistance determinants in the Comprehensive Antibiotic Resistance Database (CARD), and for Mexican *A. haemolyticus* strains, we contrasted the antibiotic resistance genes with their *in vitro* antibiograms. The antibiograms of the Mexican strains are presented in **Figure 4** and **Supplementary Table S4**.

We found that all *A. haemolyticus* genomes had an aminoglycoside acetyl-transferase gene [AAC(6′)-Ig] in the same location (in spot 5). In addition, only six strains harbored extra aminoglycoside-modifying enzymes in different locations; the majority were aminoglycoside phosphotransferases, two of which were observed in the representative genome sz1652 [APH(3″)-Ib and APH(6)-Id] in the hypervariable region between spots 7 and 8, near some conjugation proteins (TraA and TraB). This points to the role of hypervariable regions as a platform for the acquisition of novel genes by HGT.

All *A. haemolyticus* genomes had either a full-length or a fragment of a chromosomal oxacillinase, which could be $bla_{\mathrm{OXA-214}}$ or $bla_{\mathrm{OXA-215}}$ (**Supplementary Table S4**). The gene variant was not related to the position of the strain in the phylogeny (**Figure 1**). Moreover, both genes were in similar contexts (in representative strains, they were located in spot 11), and for each variant, there were point mutations in some strains but also large deletions that spanned multiple codons and resulted in truncated proteins. This could be the result of multiple mutations that occurred during independent events.

On the other hand, most *A. haemolyticus* strains isolated in Puebla (Mexico) (AN5, AN7, AN10, AN11, AN13, AN20, AN27, AN34, AN44, AN60, AN61, and AN63) and three strains from Mexico City (2227, 3281, DIV33, and 978) had a $bla_{\mathrm{TEM-116}}$ beta-lactamase, but none of the representative genomes had this gene (**Supplementary Table S4**). These observations indicated that the acquisition of $bla_{\mathrm{TEM-116}}$ occurred during multiple independent events along the phylogeny and that the insertion occurred in distinct locations in the genome.

Notably, two strains (AN54 and sz1652) had a transposon that carries an NDM-1 Metallo-beta-lactamase, which confers resistance to all beta-lactams, including carbapenems (Khan et al., 2017). In strain AN54, this gene resided in a plasmid, and in

FIGURE 4 | Antibiograms for Mexican *A. haemolyticus* strains. PIP, Piperacillin; TIC, Ticarcillin; AMC, Amoxicillin/Clavulanate; SAM, Ampicillin/Sulbactam; TZP, Piperacillin/Tazobactam; TIM, Ticarcillin/Clavulanate; CAZ, Ceftazidime; CTX, Cefotaxime; CRO, Ceftriaxone; FEP, Cefepime; IPM, Imipenem; MEM, Meropenem; ETP, Ertapenem; AZT, Aztreonam; GM, Gentamicin; AN, Amikacin; TOB, Tobramycin; TE, Tetracycline; CIP, Ciprofloxacin; LVX, Levofloxacin; SXT, Trimethoprim/Sulfamethoxazole. This data is also available in **Supplementary Table S4**.

strain sz1652, it was located in the chromosome. Additionally, in strain sz1652, there were two macrolide resistance genes (*msr*E and *mph*E) in the chromosome. This highlighted that *A. haemolyticus* is already acquiring antibiotic resistance genes of clinical concern.

The majority of the Mexican strains were susceptible to most antibiotics used to treat *Acinetobacter* infections, such as penicillins, either alone or in combination with beta-lactamase inhibitors, 3rd- and 4th-generation cephalosporins, carbapenems, monobactams, tetracyclines, fluoroquinolones, and sulfonamides (**Figure 4** and **Supplementary Table S4**). Overall, there was a decrease in susceptibility for aminoglycosides, which could be explained by the presence of different aminoglycoside-modifying enzymes. Additionally, of notable concern, there were some MDR strains resistant to three or more antibiotic families (Magiorakos et al., 2012) isolated in various years: AN3 (2010), 2227 (2011), 2126ch (2011), 3275 (2012), 3281 (2012), 10633 (2013), 5227 (2014), and AN54 (2016). However, the MDR phenotype could not be explained solely by the presence of antibiotic resistance genes because some strains harbored the same genes but were antibiotic sensitive, such as strains AN7 (antibiotic sensitive) and 3281 (MDR). Conversely, a strain could be MDR and lack some genes, such as strain 5227, which had neither extra aminoglycoside-modifying enzymes nor $bla_{\mathrm{TEM-116}}$ (**Supplementary Table S4**). Additionally, the MDR phenotype was interspersed throughout the phylogeny. All of this highlighted the relevance of HGT in the acquisition of novel genes, as well as the role of the regulation of efflux pumps, or

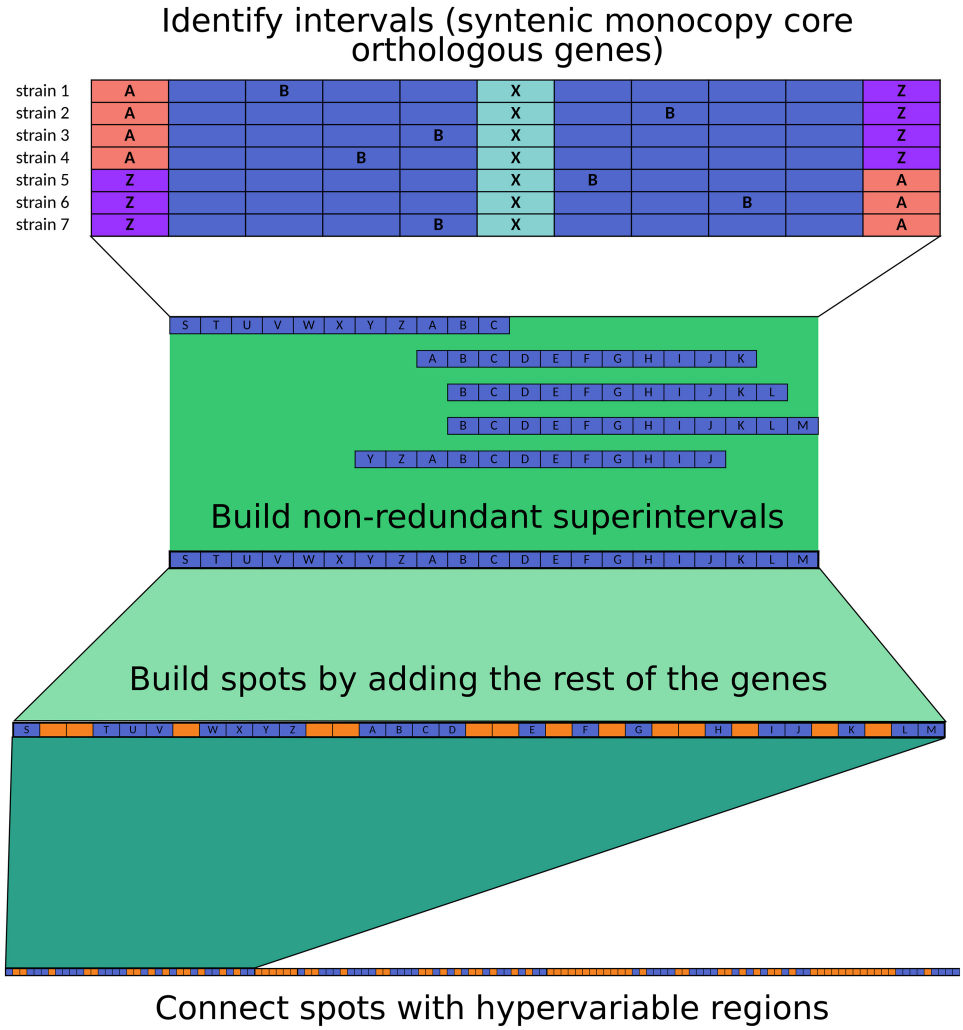

FIGURE 5 | Methodology used for spot and hypervariable region delimitation.

point mutations in either regulatory regions or genes that code for antibiotic-modifying enzymes.

Virulence Factors and Secretion Systems in all *A. haemolyticus* Strains

We also searched for protein-coding genes that were relevant for pathogens, such as virulence factors and secretion systems, in the specialized databases VFDB and TXSSCAN (Abby et al., 2014; Chen et al., 2016; Abby and Rocha, 2017) and two curated databases from the literature: one of siderophores and iron-acquisition systems and one of capsule *loci* (KLs) (see section "Materials and Methods"). Virulence factors can be grouped by function, either as immunogenic proteins, adherence factors for attachment to host and inert surfaces, capsules for protection from the environment, and siderophores and other micronutrient acquisition systems. In addition, some type-II secretion systems release effectors that damage host cells (Dobrindt et al., 2004; Wong et al., 2016; Weber et al., 2017).

The *A. haemolyticus* strains harbored multiple copies of the gene *omp*A, which codes for a porin that is one of the most abundant surface proteins in *Acinetobacter*. *A. baumannii* OmpA is immunogenic, can induce apoptosis of host cells, and is related to biofilm formation and persistence (C.-R. Wong et al., 2016; Lee et al., 2017; Weber et al., 2017). Therefore, *omp*A in *A. haemolyticus* could have the same functions and promote damage to the host. Moreover, in the representative genomes, some copies of *omp*A were in putative HGT regions defined by AlienHunter or in regions with atypical GC content, and some also had recombination signals. In addition, *omp*A copies were located in multiple spots but also in hypervariable regions. This result highlighted the high variation in this gene in *A. haemolyticus*.

The type-I secretion system (T1SS) was present in multiple copies in all *A. haemolyticus* genomes, and it formed part of the core genome. The T1SS consists of an ABC transporter, a membrane fusion protein, and an outer membrane protein (Abby and Rocha, 2017; Harding et al., 2018). In the representative

genomes, we identified two copies of the ABC transporter in spot 6, a copy of the membrane fusion protein in spot 8, and a copy of the outer membrane protein in spots 8 and 9. The T1SS exports Bap (Biofilm-associated protein) in *A. baumannii* (Harding et al., 2018), but we did not find a close homolog of this virulence factor in the *A. haemolyticus* genomes; instead, there might be other uncharacterized adhesins that are secreted by the T1SS. In addition, all strains possessed the two genes that code for BfmRS (except ATCC_19194, where only the bfmR gene was detected), a two-component system that regulates Csu (Chaperone-usher) *pili*, which are important for biofilm formation, adherence to abiotic surfaces, and capsule production (Weber et al., 2016; Lee et al., 2017). However, most of the Csu *pilus* components were annotated as hypothetical proteins (CsuA/B, CsuC, and CsuE). These results suggested that *A. haemolyticus* can form a biofilm, but the specific genes involved in its production and regulation remain to be elucidated.

All *A. haemolyticus* genomes harbor protein-coding genes for LOS biosynthesis (*lpx* genes). In the representative genomes, these genes were interspersed within spots 1, 6, 8, and 9, and almost all had recombination signals. LOSs have been implicated in serum resistance because of their role in the evasion of complement-mediated mortality and promotion of bacterial survival in host tissues (Wong et al., 2016; Geisinger et al., 2019). Importantly, in contrast to other gram-negative pathogens such as *Escherichia coli*, *Acinetobacter* members do not produce lipopolysaccharides (LPSs) because their genomes lack a ligase for LPS biosynthetic pathways (Singh et al., 2019); therefore, there are no exotoxin-like properties for these *Acinetobacter* surface structures.

The capsule is an important surface structure that in *Acinetobacter* contributes to resistance to desiccation, the ability to survive long after periods of drought, and a survival strategy linked to persistence on abiotic surfaces such as medical equipment and hospital surfaces, but it has also been linked to increased virulence, complement-mediated mortality resistance, and biofilm formation (Wong et al., 2016; Harding et al., 2018; Geisinger et al., 2019). All capsule genes in the representative genomes were located in spot 1. The KLs in *A. baumannii* exhibit great compositional variation (Harding et al., 2018); thus, it was not surprising that there were only three protein-coding genes of KLs in the core genome that also had recombination signals: *gal*U, *gdr*, and *gpi*; these genes are involved in the synthesis of oligosaccharide repeat units that will form the capsule. Notably, we also found one of the genes that commonly flanks the KLs in the core genome of *A. baumannii* (*fkp*A). The rest of the protein-coding genes involved in capsule synthesis also had recombination signals: *gne*1, *pgm*, and *ugd* (for oligosaccharide synthesis), *qhb*B (an aminotransferase), and *wza* and *wzc* (for polysaccharide export). However, we did not identify homologs of genes involved in the synthesis of the outer core of LOSs (OCL), but flanking genes (*ilv*E and *asp*S) were present (in representative genomes, they were in spot 9, such as some LOS biosynthesis genes). Among the genes flanked by *ilv*E and *asp*S, we recognized only hypothetical proteins and other enzymes annotated as having functions similar to those of the OCL, namely, an O-acetyltransferase, a capsular polysaccharide phosphotransferase, and a UDP-glucose 6-dehydrogenase. Therefore, *A. haemolyticus* produces a capsule and modifies LOSs with enzymes different from those in *A. baumannii*, but some of them are shared.

On the other hand, the type-IV pilus (T4P) is important for adhesion and twitching motility (Geisinger et al., 2019). Indeed, all strains had genes encoding twitching motility proteins (PilTU) in the same genetic context, but the regulators (PilGH) were in distinct locations. Together, these data suggested that *A. haemolyticus* can use strategies similar to those of *A. baumannii* for resistance to desiccation and survival on hospital and host surfaces.

The type-II secretion system (T2SS) can export proteins that cause host damage, for example, lipases and proteases (Weber et al., 2017; Harding et al., 2018). All strains had a complete T2SS and a phospholipase D in the same genetic context; in the representative genomes, they were located in spot 10. In addition, 10 strains (2227, 978H, AN4, CIP 64.3, KCRI-348C, MTCC 9819, NBRC 109758, NCTC 10305, NCTC 12155, and TG19599) also encoded a phospholipase C in the same genetic location, but it was present in only one representative genome (AN4) in spot 1. This finding highlighted that *A. haemolyticus* can produce a variety of enzymes that are important for the acquisition of nutrients and lysis of host cells.

Hosts can inhibit bacterial growth by sequestration of iron; however, to overcome this limitation, bacteria harbor iron-transport and iron-acquisition systems (Antunes et al., 2011). To date, the following systems have been described in *Acinetobacter*: an ABC-transporter system for ferrous iron (FeoABC) (Antunes et al., 2011); at least three siderophore clusters for iron acquisition: acinetobactin (Zimbler et al., 2009; Hasan et al., 2015), baumanoferrin (Antunes et al., 2011; Eijkelkamp et al., 2011; Penwell et al., 2015), and enterobactin (Dorsey, 2003; Antunes et al., 2011); and two clusters for hemin uptake, used to sequester iron from the host (Antunes et al., 2011). FeoABC was located in the core genome and, in the representative genomes, was located in spot 1; feoB and feoC had recombination signals. Consequently, FeoABC can serve as a platform for the acquisition of novel genes by recombination if the cluster is not lost.

Fourteen strains (AN43, AN59, ATCC 19194, ATCC 27244, CIP 64.3, HW-2A, MTCC 9819, NBRC 109758, NCTC 10305, NCTC 10306, NCTC 12155, NIPH 61, TG19599, and TG21157) contained most of the protein-coding genes needed for the biosynthesis and transport of acinetobactin (14 to 16 of 18 genes); in three representative genomes (AN43, AN59, and HW-2A), this cluster was located in spot 8, but it was absent in the rest of the genomes. Inside this region in strains AN43 and AN59, signals of HGT were detected by AlienHunter and two IS66 transposases, whereas in strain HW-2A, we detected only unique gene families. These data showed that the acinetobactin biosynthesis cluster had a foreign origin, as is the case in *A. baumannii* (Antunes et al., 2011).

Genes involved in enterobactin synthesis were not found; therefore, *A. haemolyticus* might not produce this siderophore.

Only a few genes for the synthesis of baumanoferrin were present (2 or 3 of 11), and two of them were in the core genome; in the representative genomes, all were located in spot 6. Therefore, *A. haemolyticus* might produce neither enterobactin nor baumanoferrin but have the potential to acquire the genes necessary for their production or the production of novel siderophores. Alternatively, this species might already produce them, in which case their characterization is pending.

All but 6 strains (AN60, AN61, AN63, HW-2A, KCRI-348C, and TJS01) harbored almost all the components of a hemin cluster (10 or 11 of 11 genes), but only one gene was located in the core genome (ACICU_RS08290). In the representative genomes, these genes were located in spot 4 (but one gene was located in spot 6), and only two genes had recombination signals. On the other hand, all but 9 strains (11654, ATCC 19194, CIP 64.3, MTCC 9819, NBRC 109758, NCTC 10305, NCTC 12155, TG19599, and XH900) contained almost all the components of another hemin cluster (5 to 7 of 8 genes). These genes were also located in spot 6 in the representative strains, and two of them had recombination signals (ACICU_RS04580 and ACICU_RS04605). All these data showed that *A. haemolyticus* can uptake hemin for iron acquisition and that the gene clusters had been integrated via recombination with neighboring genes.

Regarding virulence factors characterized in other gram-negative bacteria, we found that all strains had a superoxide dismutase (*sod*) and that all but three strains (11616, 3281, and KCRI-348C) had a gene that codes for a catalase/peroxidase (*kat*B); both enzymes provide resistance to reactive oxygen species-induced killing via immune system cells (Heindorf et al., 2014; Harding et al., 2018). The *sod* gene can be flanked by different genes, even though in the representative genomes it was always located in spot 5, however, the gene had recombination signals, and in a couple of strains (5227 and AN54), AlienHunter detected it as a region of putative foreign origin. *kat*B was located in spot 2 in the representative genomes and in similar genetic contexts in the rest of the genomes, but there was also slight variation in gene content in this region; this could also be explained by the fact that the *kat*B gene also had recombination signals.

Finally, TXSSCAN identified two elements of the flagellum, but they were false positives because *Acinetobacter* does not have flagella. Indeed, the elements identified were a transcriptional terminator and a subunit of an ATP synthase, which could have distinct functions in the biology of this organism.

In general, in the *A. haemolyticus* genomes, we found most of the virulence factors previously characterized in *A. baumannii* but also signs of the foreign origin of these systems. However, some elements were absent in these genes, even if the strains were associated with human infections. This can be explained by the "damage-response framework," which highlights that virulence is mediated by bacterium, host characteristics and other factors (Casadevall and Pirofski, 2019). The patients' clinical data showed that their health was compromised because of immunosuppression or because host barriers were broken by invasive procedures; thus, it is possible that the presence of additional virulence factors exacerbated the infection in most susceptible hosts.

CONCLUSION

In this work, we analyzed 47 genomes of the opportunistic pathogen *A. haemolyticus*, 31 of which were contributed by us. We found that multiple lineages of *A. haemolyticus* are circulating in Mexican and Chinese hospitals and that Mexican strains are more closely related than strains isolated from other countries. We also pointed out that the *A. haemolyticus* chromosome is fragmented into large syntenic regions (spots) and hypervariable regions that can span one to hundreds of genes. Most of the core monocopy orthologs lie in spots, many of which have recombination signals and thus serve as receptors of novel genes introduced by homologous recombination. Hypervariable regions are platforms of gene acquisition, for example, mediated by transposition. Finally, we found that *A. haemolyticus* strains are already acquiring antibiotic resistance determinants and virulence factors, which may complicate treatment and exacerbate illness in infected hosts. The virulence factors were located only in chromosomes, but as antibiotic resistance determinants are located in both chromosomes and plasmids, surveillance and analysis of *A. haemolyticus* plasmids are also warranted.

DATA AVAILABILITY STATEMENT

The genome assembly accession numbers are listed in **Table 1** and the reads are listed in **Supplementary Table S1**.

ETHICS STATEMENT

The protocol for this study was approved by the Committee of Hospital para el Niño Poblano (Registry Number: HNP/ENS/177/2016), the committee waived the need for written informed consent from patients.

AUTHOR CONTRIBUTIONS

SC-J contributed to the initial species designation by *rpoB*, genome assembly, bioinformatic analyses, data visualization, and manuscript writing. EB-L helped with the initial species designation by *rpoB* and antibiograms. CV-A performed the antibiograms and provided the clinical data. PV-F and PL-Z provided isolates, clinical data, and resources. SC-R gave feedback for some bioinformatic analyses, and manuscript editing. MC designed the study, acquired the funding, and wrote and edited manuscript.

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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.2020.00926/full#supplementary-material

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Resultados adicionales

Perfiles de plásmidos

Las cepas clínicas mexicanas de *A. haemolyticus* (resaltadas en anaranjado en las Figuras 2 a 5) tienen variación en la cantidad y el tamaño de sus plásmidos. Los perfiles se obtuvieron mediante electroforesis en gel horizontal (Eckhardt, 1978; Hynes and McGregor, 1990).

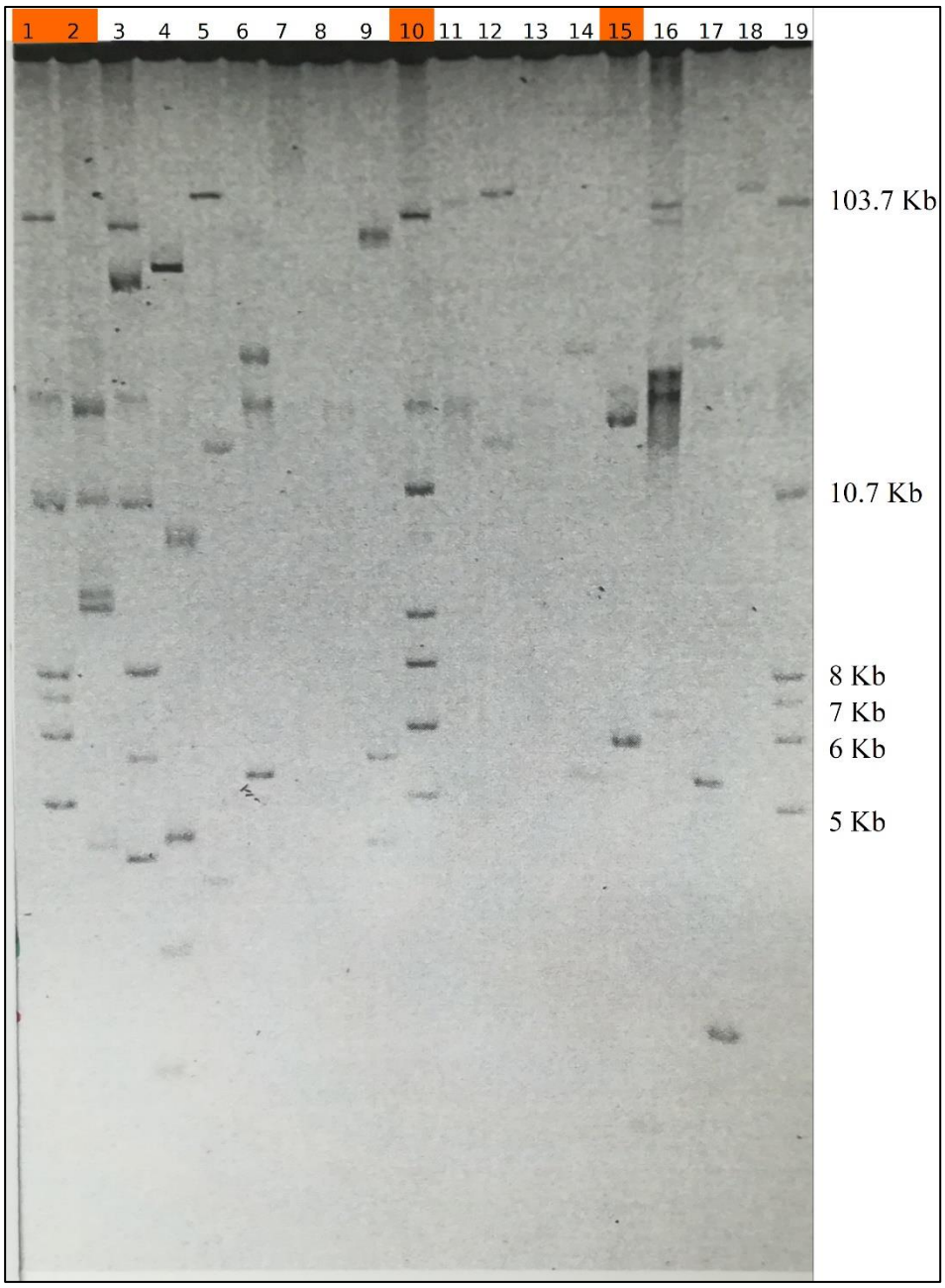

Figura 2. Perfil de plásmidos de cepas de *Acinetobacter*, aisladas de pacientes del Instituto Nacional de Cancerología.

En anaranjado se resaltan los carriles con cepas de *A. haemolyticus*. Los nombres de las cepas son: 11616 (1), 2227 (2), 2126ch (3), 2126gde (4), 2294 (5), 2161 (6), 2490 (7), 2592 (8), 2339 (9), 3281 (10), 3108 (11), 3071 (12), 3496 (13), 5189 (14), 5227 (15), 4844gde (16), 6438ch (17), 6793 (18), 11616 (19; control).

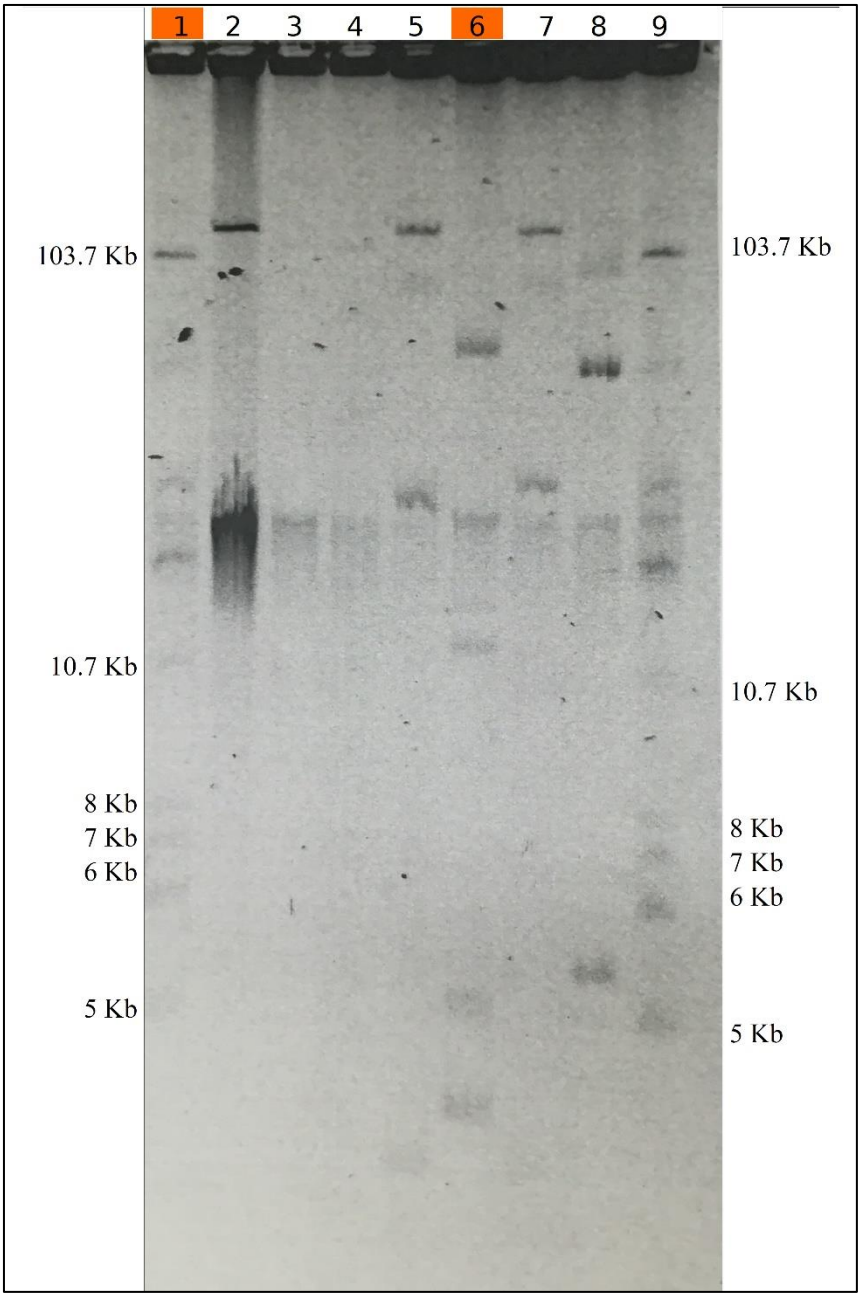

Figura 3. Perfil de plásmidos de cepas de *Acinetobacter*, aisladas de pacientes del Instituto Nacional de Cancerología.

En anaranjado se resaltan los carriles con cepas de *A. haemolyticus*. Los nombres de las cepas son: 11616 (1; control), 2275 (2), 6793 (3), 3108gde (4), 3071gde (5), 3275 (6), 2294 (7), 2663 (8), 11616 (9; control).

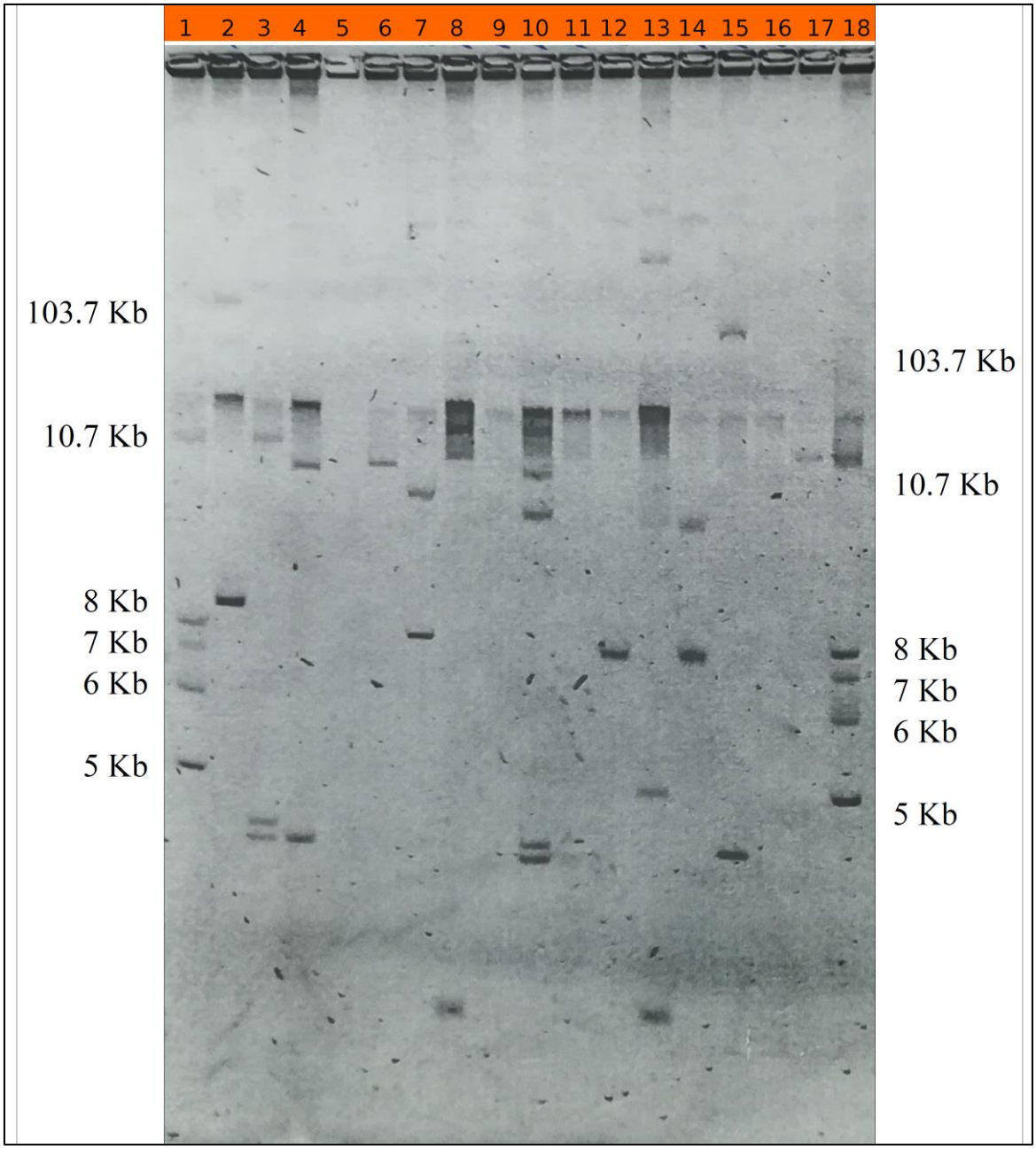

Figura 4. Perfil de plásmidos de cepas de *Acinetobacter haemolyticus*, aisladas de pacientes del Hospital del Niño Poblano.

En anaranjado se resaltan los carriles con cepas de *A. haemolyticus*. Los nombres de las cepas son: 11616 (1; control), AN3 (2), AN4 (3), AN5 (4), AN7 (5), AN10 (6), AN11 (7), AN13 (8), AN20 (9), AN27 (10), AN34 (11), AN43 (12), AN44 (13), AN59 (14), AN60 (15), AN61 (16), AN63 (17), 11616 (18; control).

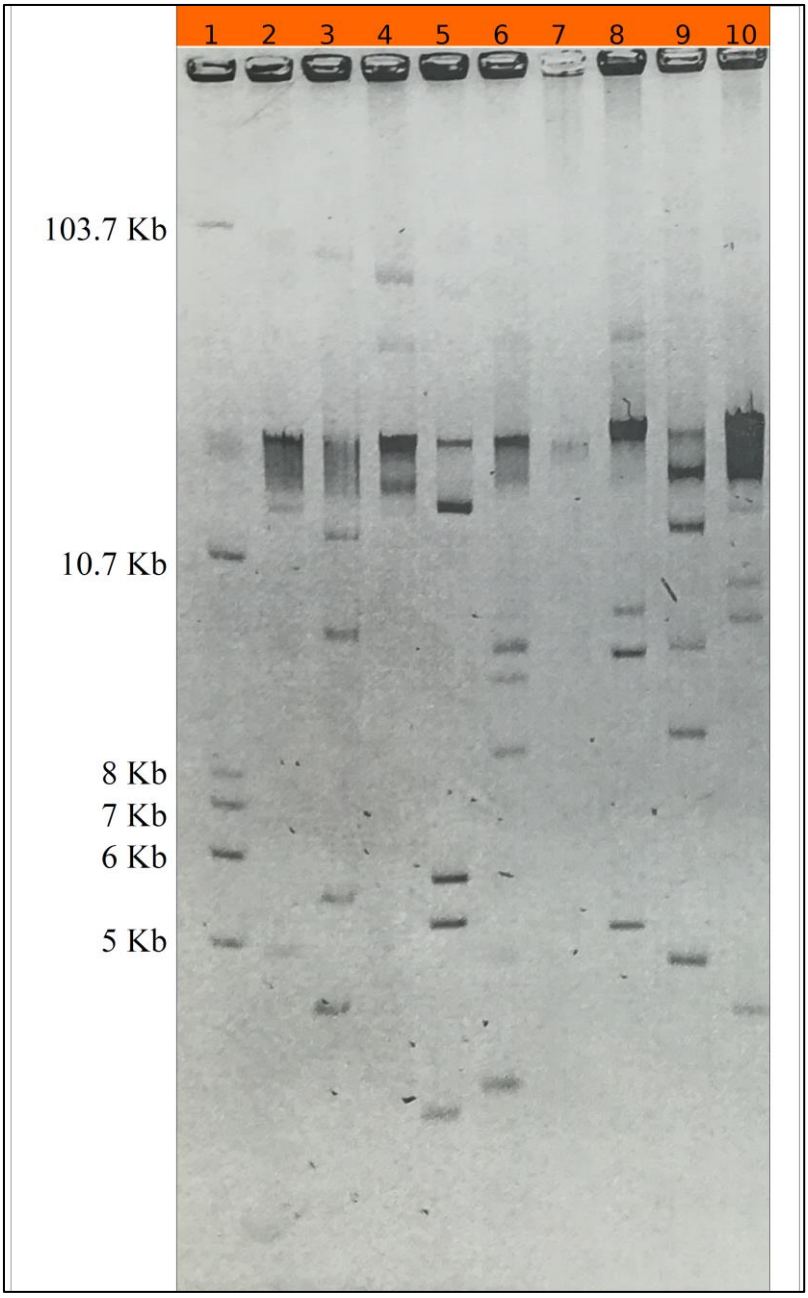

Figura 5. Perfil de plásmidos de cepas de *Acinetobacter haemolyticus*, aisladas de pacientes de diversos hospitales.

En anaranjado se resaltan los carriles con cepas de *A. haemolyticus*. Los nombres de las cepas son: 11616 (1; control), 10633 (2), 5439 (3), 11650 (4), 11654 (5), 11658 (6), 11652 (7), INNSZ174 (8), AN54 (9), DIV33 (10).

Conclusiones

A. haemolyticus es un patógeno oportunista que puede ser más problemático si aumenta la frecuencia de cepas multi-drogo resistentes, ya que los determinantes de resistencia le permitirán prevalecer en los hospitales como a *A. baumannii*, pues las cepas de *A. haemolyticus* circulantes ya cuentan con distintos factores de virulencia que le permitirían causarle daño a su hospedero, y también tienen genes relacionados con la biosíntesis de la cápsula, una envoltura de polisacáridos que puede proteger a las bacterias de la desecación. Sin embargo, a nivel de genoma completo, *A. baumannii* y *A. haemolyticus* son muy diferentes: son claramente especies distintas, y se puede ver esta distinción con métodos globales como el nivel de identidad promedio a lo largo del genoma (ANI), con una filogenia hecha a partir de los genes que codifican para proteínas ribosomales, ya que muestran que ambas especies sí están relacionadas porque pertenecen al mismo género, pero no son muy cercanas, e incluso se encuentran en distintos clados), con las diferencias en contenido génico, y por la filogenia hecha a partir de genes ortólogos compartidos.

Las 44 cepas de *A. haemolyticus* estudiadas, cuyas fechas de aislamiento abarcan desde los años sesenta hasta el 2017, se encuentran repartidas en distintos clados a lo largo de una filogenia hecha a partir de los genes ortólogos compartidos entre todos los genomas, sin señales de recombinación. En México y en China hay distintos linajes circulando en sus hospitales, que no parecieran estar asociados a brotes epidémicos. Algunas cepas aisladas en México son más parecidas entre sí, a pesar de provenir de hospitales distintos y haber sido aisladas en años diferentes. Las cepas aisladas de China y Tanzania son las que más se distinguen de todo el conjunto de cepas.

El cromosoma de *A. haemolyticus* está conformado por doce regiones que comparten los mismos genes ortólogos y donde se conserva el orden relativo de los mismos (*spots*). Sin embargo, los genes accesorios reflejan una gran variación en el contenido génico incluso dentro de los *spots*. La variación en el contenido de genes se ve más reflejada en las regiones hipervariables, que separan a los *spots* en algunas cepas, y que en algunas otras cepas no existen; en estos últimos casos los *spots* se encuentran de manera contigua, sin separación. Las regiones hipervariables pudieran ser plataformas de adquisición de genes

mediada por, por ejemplo, transposición, especialmente por la existencia de transposasas asociadas a secuencias de inserción en muchas de las regiones hipervariables.

Varias de las cepas de *A. haemolyticus* analizadas cuentan con varios genes que codifican para factores de virulencia en el cromosoma. Un par de cepas portan un determinante de resistencia muy problemático en los hospitales, la metalo-beta-lactamasa NDM-1, que confiere resistencia a los carbapenémicos, antibióticos de elección para el tratamiento de infecciones causadas por las *Acinetobacter*, y se encuentra asociada a un transposón, y que en una cepa está en el cromosoma y en otra en un plásmido.

Perspectivas

El presente estudio se enfocó en el análisis del cromosoma de *A. haemolyticus*, así que los análisis posteriores se podrían orientar hacia los plásmidos, especialmente dada la variación en cantidad y tamaño de estos, tal como reflejan los geles horizontales de perfiles de plásmidos en las cepas mexicanas. Por ejemplo, se podría comparar a los plásmidos de *A. haemolyticus* con los de *A. baumannii* y hacer experimentos para probar si los plásmidos de una especie pueden replicar en la otra, y en caso afirmativo, qué tan bien pueden coexistir con plásmidos propios de cada especie, y establecer grupos de incompatibilidad. Además, se podría comparar a los plásmidos de *A. haemolyticus* con los de otras especies de relevancia médica o con los de cepas aisladas del ambiente, para determinar si los plásmidos de *A. haemolyticus* son más parecidos a los de bacterias de los hospitales o a los que se encuentran en las cepas ambientales, o si presentan una combinación de ambas fuentes. Asimismo, se podría aislar y caracterizar por separado los plásmidos de *A. haemolyticus* para entender su biología, e incluso modificarlos y aprovecharlos como vectores, pues hay pocos plásmidos de uso rutinario que funcionen para el género *Acinetobacter*, a diferencia de organismos muy trabajados en biología molecular como *Escherichia coli*.

Si se secuenciaran los genomas de varias cepas de *A. haemolyticus* aisladas entre 1960 y 2011, que es una ventana de tiempo poco cubierta por este estudio, se podrían hacer análisis bayesianos de datación para determinar las fechas de introducción a distintos países, el surgimiento de linajes importantes, o de adquisición de ciertos genes.

Hacen falta estudios para identificar al gen o genes asociados al fenotipo de hemólisis, pues *A. haemolyticus* recibe su nombre por tener actividad hemolítica, ya que la mayoría de las cepas tienen ese fenotipo. Además, dado que algunas bacterias que hacen hemólisis son más virulentas, se podría estudiar si esto es cierto para las *A. haemolyticus* u otro de los miembros del clado hemolítico, y si las cepas que carezcan del fenotipo de hemólisis están asociadas a infecciones menos agresivas.

No hay una perfecta correlación entre portar genes de resistencia a antibióticos y tener el fenotipo de resistencia, pues es posible que las bombas de eflujo confieran resistencia a los

antibióticos sin que esté presente un gen específico asociado a dicha resistencia. Sin embargo, es importante considerar las redes de regulación genética y si los genes asociados a resistencia a los antibióticos cuentan con un promotor funcional, ya que pudiera ser que el gen se encuentre presente en el genoma, pero no se exprese, o que su nivel de expresión sea insuficiente para conferirle resistencia a la bacteria. Asimismo, es posible que las proteínas producidas tengan menor actividad, pues casi todos los genes de resistencia a antibióticos tenían diferencias con respecto a los alelos descritos en las bases de datos.

Finalmente, los factores de virulencia encontrados *in silico* podrían ser validados en modelos animales como larvas y ratones, y en cultivos de células humanas. De esta manera, con modelos de infección establecidos y mediante estudios de mutagénesis aleatoria mediada por transposones, se podría identificar a más genes relacionados con la virulencia.

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Apéndice 1 - Complete Genome Sequence of a Multidrug-Resistant *Acinetobacter baumannii* Isolate Obtained from a Mexican Hospital (Sequence Type 422).

Castro-Jaimes, S., Salgado-Camargo, A. D., Graña-Miraglia, L., Lozano, L., Bocanegra-Ibarias, P., Volkow-Fernández, P., et al. (2016). Complete Genome Sequence of a Multidrug-Resistant *Acinetobacter baumannii* Isolate Obtained from a Mexican Hospital (Sequence Type 422). *Genome Announc.* 4, e00583-16. doi:10.1128/genomeA.00583-16.

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Complete Genome Sequence of a Multidrug-Resistant *Acinetobacter baumannii* Isolate Obtained from a Mexican Hospital (Sequence Type 422)

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***Acinetobacter baumannii* has emerged as a dangerous nosocomial pathogen, particularly for severely ill patients in intensive care units and patients with hematologic malignancies. Here, we present the complete genome sequence of a multidrug-resistant *A. baumannii* isolate, recovered from a Mexican hospital and classified as sequence type 422 according to the multilocus sequence typing Pasteur scheme.**

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Acinetobacter baumannii is a Gram-negative bacterium that has emerged as a dangerous pathogen worldwide, causing a variety of nosocomial infections, especially in severely ill patients in intensive care units and patients with hematologic malignancies on chemotherapy (1). *A. baumannii* isolates are resistant to most of the currently used antibiotics, including carbapenems, and they are able to survive for extended periods of time under a broad range of environmental conditions, such as dry surfaces. These characteristics promote *A. baumannii* outbreaks in the hospital setting. Most of the isolates causing outbreaks worldwide belong to the international clonal lineages I and II (2), clonal complexes 1 and 2, respectively, as defined by the multilocus sequence typing (MLST) Pasteur scheme (3).

Here, we present the complete genome sequence and annotation of *A. baumannii* 3207, an isolate not related to the international clonal lineages but instead classified as sequence type 422 according to the MLST Pasteur scheme (3). This multidrug-resistant isolate was recovered from bronchoalveolar lavage fluid of a 45-year-old female patient admitted to the Hospital Universitario de Nuevo León, Mexico, a tertiary care center, in August 2008.

The complete genome of *A. baumannii* 3207 was determined on a PacBio RSII platform. To do this, a large insert library (20 to 25 kb) was constructed and sequenced, using one SMRT cell with a P6 polymerase and C4 chemistry combination (P6-C4) with a 180-min movie. The SMRT cells produced 551,632,314 post-filter polymerase reads with a mean read length of 10,718 bp. Subreads were assembled *de novo* using the PacBio RS hierarchical genome assembly process (HGAP) protocol version 3 in SMRT analysis version 2.3 (Pacific Biosciences) (4). To improve regions of low coverage, a library with a 2 × 300-bp paired-end configuration was sequenced on an Illumina MiSeq platform. The sequencing yielded 3,117,310 reads, resulting in 909,042,928 bp. A hybrid assembly was constructed with the Illumina MiSeq reads and PacBio RII subreads using SPAdes version 3.5.0 (5). The contigs corresponding to the chromosome and the plasmids were circularized using a Perl script available at https://github.com/jfass/apc. Functional annotation was performed with the NCBI Prokaryotic Genome Annotation Pipeline.

The *A. baumannii* 3207 genome consists of a circular chromosome (3,998,013 bp) and two plasmids: pAba3207a (13,479 bp) and pAba3207b (80,547 bp). The chromosome has six rRNA operons, 74 tRNAs, and 3,674 coding sequences (CDSs) and embraces most of the antibiotic resistance genes, conferring resistance to aminoglycosides, beta-lactams, carbapenems (blaOXA-65), cephalosporins, fosmidomycin, fusaric acid, phenicols, and tetracyclines. The smallest plasmid has 19 CDSs, including one carbapenem resistance gene (blaOXA-58), whereas pAba3207b has 97 CDSs.

This and a previous report (6) are the first steps toward understanding the genomic diversity of *A. baumannii* in Mexico, not only at some loci but at the genome level. However, to get a more comprehensive picture, comparative analyses of many more isolates are warranted.

Nucleotide sequence accession numbers. The genome sequence of isolate 3207 was deposited in GenBank under the accession numbers CP015364, CP015365, and CP015366.

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PacBio sequencing was performed at the Duke GCB Genome Sequencing Shared Resource (Durham, NC, USA). Illumina sequencing was conducted at Macrogen, (Seoul, South Korea).

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Apéndice 2 - First Genome Sequence of a Mexican Multidrug-Resistant *Acinetobacter baumannii* Isolate.

Graña-Miraglia, L., Lozano, L., Castro-Jaimes, S., Cevallos, M. A. M. A., Volkow, P., and Castillo-Ramírez, S. (2016). First Genome Sequence of a Mexican Multidrug-Resistant *Acinetobacter baumannii* Isolate. *Genome Announc.* 4, 4–5. doi:10.1128/genomeA.00156-16.

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First Genome Sequence of a Mexican Multidrug-Resistant *Acinetobacter baumannii* Isolate

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***Acinetobacter baumannii* has emerged as an important nosocomial pathogen worldwide. Here, we present the draft genome of the first multidrug-resistant *A. baumannii* isolate, sampled from a tertiary hospital in Mexico City. This genome will provide a starting point for studying the genomic diversity of this species in Mexico.**

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Acinetobacter baumannii, a Gram-negative coccobacillus, has surfaced as one of the leading causes of hospital-acquired infections in recent years (1, 2). Remarkably, many strains of this species have multidrug-resistant phenotypes showing resistance against most antibiotic classes (2). Ever since the publication of the first genome sequence of an *A. baumannii* isolate a considerable amount of genome sequences have been published. As of 23 November 2015, there are slightly over 30 complete genomes and more than a thousand draft genomes publically available. However, none of them has come from Mexico. Here, in order to start exploring the genome diversity of this species in Mexico, we report the first genome sequence of the Mexican isolate *A. baumannii* Mex11594, hereinafter referred to as Mex11594.

Mex11594 was isolated on 17 June 2011 at the Instituto Nacional de Cancerología (Mexico's National Institute of Oncology), a tertiary hospital located in Mexico City, from pleural fluid of a male patient. Multilocus sequence typing (the Oxford scheme) on this strain revealed that it belongs to the sequence type (ST) 231 (3). According to the PubMLST database (4), isolates sharing this ST have been found in Argentina, Brazil, France, Germany, the Netherlands, and United States, implying that this ST is not exclusive to Latin America. The Mex11594 genome was sequenced using an Illumina MiSeq platform with a 300-bp library (250-bp paired-end reads). The sequencing run produced 1,631,060 reads that were assembled into a preliminary assembly using Velvet version 1.2.10 (5) and SPAdes (6). In order to improve our assembly, we used Consed version 23.0 (7) for gap closure, assembly editing, and error correction. The improved assembly yielded 12 contigs and had a total size of 3,745,499 bp, with an N_{50} of 684,685 bp, an average coverage of 41× and a G+C content of 39.03%. Given the number of contigs, the N_{50}, and the average coverage, we are confident that this is a good assembly that should be helpful for future comparative genome analysis. Our improved assembly suggested the existence of several plasmids, which was corroborated via an Eckhardt gel analysis (8). The chromosome was composed of 8 contigs and had an estimated size of 3,700,249 bp.

The draft genome was annotated by means of the Rapid Annotations Using Subsystems Technology (RAST) (9) server, which predicted 3,488 coding sequences, 772 of them hypothetical proteins. Ten rRNAs and 51 tRNAs were identified. This is a multidrug-resistant isolate (10), as it has molecular determinants conferring resistance to at least 3 different types of antibiotics. For instance, this isolate is nonsusceptible to meropenem and imipenem (carbapenems), tobramycin (aminoglycosides), levofloxacin (fluoroquinolones), and ceftazidime and ceftriaxone (cephalosporins) to name but a few.

Further and more extensive comparative analysis of this and other Mexican isolates will help to unveil the genomic diversity (as well as the genetic components conferring the multidrug-resistant phenotypes) of this bacterial pathogen in Mexico and Latin America and, hence, to better understand its worldwide diversity.

Nucleotide sequence accession number. The *A. baumannii* MEX11594 genome sequence was deposited in GenBank under the accession number LQXZ00000000.

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Apéndice 3 - Complete Genome Sequence of a *bla*$_{\text{OXA-58}}$-Producing *Acinetobacter baumannii* Strain Isolated from a Mexican Hospital.

Pérez-Oseguera, Á., Castro-Jaimes, S., Salgado-Camargo, A. D., Silva-Sanchez, J., Garza-González, E., Castillo-Ramírez, S., et al. (2017). Complete Genome Sequence of a bla OXA-58 -Producing *Acinetobacter baumannii* Strain Isolated from a Mexican Hospital. *Genome Announc.* 5, e00949-17. doi:10.1128/genomeA.00949-17.

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Complete Genome Sequence of a *bla*$_{OXA-58}$-Producing *Acinetobacter baumannii* Strain Isolated from a Mexican Hospital

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ABSTRACT In this study, we present the complete genome sequence of a *bla*$_{OXA-58}$-producing *Acinetobacter baumannii* strain, sampled from a Mexican hospital and not related to the international clones.

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Acinetobacter baumannii is a pathogen responsible for numerous infections and outbreaks in the clinical environment, and it is a major cause of morbidity and mortality in Latin America and in the rest of the world (1, 2). The abilities of this organism to acquire antibiotic resistance genes, to form biofilms, and to resist desiccation facilitate its permanence in the hospital setting and promote the emergence of outbreaks. Most of the nosocomial outbreaks worldwide are produced by a limited group of strains belonging to the international clones I and II (3).

In the last decade, this organism has steadily increased its resistance to carbapenems, an alarming situation because those antibiotics are one of the last-resort drugs for treating infections. Resistance to carbapenems in *A. baumannii* has been linked to the production of six groups of carbapenem-hydrolyzing class D β-lactamases: OXA-51-like, OXA-23-like, OXA-40/24-like, OXA-58-like, OXA-143-like, and OXA-235-like. Genes encoding OXA-51-like carbapenemases are encoded in the chromosome of almost all *A. baumannii* strains. The other five groups of carbapenemases are usually encoded within mobile genetic elements such as plasmids and transposons (4, 5).

A. baumannii strain 7804 was recovered in July 2006 from a bronchoalveolar lavage fluid specimen from a 25-year-old male patient admitted to the Hospital Universitario de Nuevo León, a tertiary care center (Nuevo León State, Mexico). A previous study showed that this strain is not susceptible to carbapenems and is not related to the international clones; it belongs to sequence type 490 (ST490) (Oxford scheme) and to clonal complex 110 (CC110) (6). The same study also indicated that this strain has an OXA-58-like gene associated with an ISAba3 element (6).

The genome sequence of this strain was determined with two single-molecule real-time (SMRT) cells on a PacBio RSII platform. Subreads were assembled *de novo* using the RS hierarchical genome assembly process (HGAP) protocol version 3, in SMRT analysis version 2.3 (Pacific Biosciences). The assembly has 98× coverage. Unitigs corresponding to the chromosome and plasmids were circularized using a Perl script (available at https://github.com/jfass/apc). Functional annotation was done with the NCBI Prokaryotic Genome Annotation Pipeline. The genome of *A. baumannii* 7804 contains one circular chromosome (4,159,217 bp) and two plasmids, pAba7804a (12,381 bp) and pAba7804b (170,420 bp). The chromosome has 6 rRNA operons, 75 tRNAs, and 3,892

coding sequences (CDS). The acquired antibiotic resistance genes were identified via ResFinder 2.1 (7) with the following results: the chromosome embraces one gene encoding aminoglycoside resistance *aph*(*3″*)-*Via* and two genes encoding β-lactamases genes (bla_{ADC-25} and bla_{OXA-64}). Plasmid pAba7804b carries two genes related to aminoglycosid resistance (*strA* and *strB*), one sulfonamide resistance gene (*sul2*), and another gene related to tetracycline resistance (*tetB*). The bla_{OXA-58} gene is carried in the small plasmid. Two recently published genomes (8, 9) along with this strain represent the first *A. baumannii* genome sequences from Mexico and should be instrumental in the characterization of the genomic diversity of this nosocomial pathogen in this country.

Accession number(s). The genome sequence of isolate 7804 was deposited in GenBank under the accession numbers CP022283, CP022284, and CP022285.

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Apéndice 4 - Resistome and a Novel *bla*$_{\text{NDM-1}}$-Harboring Plasmid of an *Acinetobacter haemolyticus* Strain from a Children's Hospital in Puebla, Mexico.

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Resistome and a Novel *bla*$_{NDM-1}$-Harboring Plasmid of an *Acinetobacter haemolyticus* Strain from a Children's Hospital in Puebla, Mexico

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Acinetobacter calcoaceticus-baumannii complex isolates have been frequently associated with hospital and community infections, with *A. baumannii* being the most common. Other *Acinetobacter* spp. not belonging to this complex also cause infections in hospital settings, and the incidence has increased over the past few years. Some species of the *Acinetobacter* genus possess a great diversity of antibiotic resistance mechanisms, such as efflux pumps, porins, and resistance genes that can be acquired and disseminated by mobilizable genetic elements. By means of whole-genome sequencing, we describe in the clinical *Acinetobacter haemolyticus* strain AN54 different mechanisms of resistance that involve *bla*$_{OXA-265}$, *bla*$_{NDM-1}$, *aphA6*, *aac(6')-Ig*, and a resistance-nodulation-cell division-type efflux pump. This strain carries six plasmids, of which the plasmid pAhaeAN54e contains *bla*$_{NDM-1}$ in a Tn125-like transposon that is truncated at the 3′ end. This strain also has an insertion sequence IS*91* and seven genes encoding hypothetical proteins. The pAhaeAN54e plasmid is nontypable and different from other plasmids carrying *bla*$_{NDM-1}$ that have been reported in Mexico and other countries. The presence of these kinds of plasmids in an opportunistic pathogen such as *A. haemolyticus* highlights the role that these plasmids play in the dissemination of antibiotic resistance genes, especially against carbapenems, in Mexican hospitals.

Keywords: *A. haemolyticus*, plasmid, NDM-1, antibiotic resistance

Introduction

The genus *Acinetobacter* includes a group of bacteria that can be isolated from a wide variety of environmental sources, including soil and water. However, some of them have become important nosocomial pathogens, such as those included within the *Acinetobacter calcoaceticus-baumannii* complex. Members of this genus have been associated with severe nosocomial and community infections with high mortality rates. Nevertheless, isolates of other non-*baumannii* spp. have gained medical relevance because of their increased frequency in recent years, for example, *Acinetobacter haemolyticus*, *Acinetobacter lwoffii*, *Acinetobacter ursingii*, *Acinetobacter parvus*, and *Acinetobacter junii*.[1–3]

Acinetobacter spp. have developed resistance to multiple classes of antimicrobial agents, including broad-spectrum cephalosporins, carbapenems, fluoroquinolones, and aminoglycosides. This resistance is due to multiple mechanisms, such as resistance-nodulation-cell division (RND)-type efflux pumps, CarO porin, and resistance genes. In addition, the ability of *Acinetobacter* spp. to acquire mobilizable

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elements that carry antibiotic resistance genes increases the resistance dissemination.[4–6] In particular, bla_{NDM-1}, which encodes the New Delhi Metallo-β-lactamase-1 (NDM-1), hydrolyzes a broad spectrum of β-lactam antibiotics, including carbapenems, and is among the most worrisome resistance determinants that have spread around the world, severely complicating the treatment of nosocomial infections.[7–10] In Latin America, its presence has been reported in Enterobacteriaceae isolates such as *Klebsiella pneumoniae*, *Escherichia coli*, and *Providencia rettgeri*,[11–13] and in nonfermentative bacilli, such as *A. baumannii*, *Acinetobacter pittii*, *Acinetobacter bereziniae*, and *A. haemolyticus*.[14–17] In this work, we report the presence of diverse antibiotic resistance genes in a strain of *A. haemolyticus* obtained from a Mexican pediatric patient and the characterization of the complete plasmid carrying bla_{NDM-1} isolated from this strain.

Materials and Methods

Bacterial isolation

Acinetobacter haemolyticus AN54 was recovered from peritoneal dialysis fluid culture from a 12-year-old male patient who had been admitted to the hospital for end-stage renal disease in February 2016. The patient was previously treated with ceftriaxone (CRO) and trimethoprim-sulfamethoxazole (SXT). The isolate was identified with the VITEK 2 system (bioMérieux) and molecular typing by sequencing of the *rpoB* gene.[18,19]

Antimicrobial susceptibility testing

An antimicrobial susceptibility test was performed by using the agar disk diffusion method according to the Clinical and Laboratory Standards Institute guidelines.[20] The following antimicrobials were tested: piperacillin (PIP), ticarcillin (TIC), ampicillin/sulbactam, piperacillin/tazobactam, ticarcillin-clavulanic acid (TIM), ceftazidime (CAZ), cefepime (FEP), cefotaxime (CTX), CRO, imipenem (IPM), meropenem (MEM), gentamicin, amikacin (AN), tetracycline, ciprofloxacin, levofloxacin, and SXT. The minimal inhibitory concentration (MIC) for CTX, CAZ, FEP, MEM, IPM, and AN was determined by the agar dilution method.[20] Metallo-β-lactamase (MBL) detection was performed by IPM and MEM disks supplemented with 10 μL of 0.5 M ethylenediaminetetraacetic acid.[21]

Test of the activity of the efflux pump in antibiotic resistance

The activity of the efflux pump was evaluated by using phenylalanine-arginine β-naphthylamide (Sigma-Aldrich) as an efflux pump inhibitor (EPI). The test was performed as follows: MICs for AN, CTX, and MEM were determined by the agar dilution method in the presence and absence of EPI (25 mg/L). A twofold or greater decrease in MIC in the presence of EPI was considered indicative of a role of RND-type efflux pumps in the resistance to the antibiotics tested. Test veracity was checked by using the strain *Acinetobacter haemolyticus* HNP11 as a positive control. To evaluate the effect of EPI on bacterial growth, all bacteria were cultured in Mueller–Hinton broth with and without EPI (25 mg/L).[22]

Whole-genome sequencing and data analysis

A high-quality draft genome sequence from isolate AN54 was obtained by using an Illumina MiSeq platform (2 × 300 paired-end reads) (IBT-UNAM) and one SMRT cell of PacBio RS II system (Yale Center for Genome Analysis). With data obtained from both platforms, a hybrid assembly was performed with the Unicycler assembler version 0.4.1.[23] and SPAdes version 3.11.1.[24] ResFinder 2.1[25] was used to identify and determine the location of antibiotic resistance genes. MAUVE version 20150226,[26] CLC Sequence Viewer 8.0 and BLAST were used to align and compare sequences. EASYFIG 2.2.2 was used to draw figures.[27]

Pulsed-field gel electrophoresis and Southern blot

The S1 nuclease-pulsed-field gel electrophoresis (PFGE) method was carried out to determine the plasmids number by using the *Escherichia coli* strain NCTC 50192 as a reference. To detect the resistance gene in the plasmid, the PFGE gel was transferred to a nylon membrane (Hybond-N; GE Healthcare Life Sciences), and hybridization was performed with the Dig-High Prime DNA Labeling and Detection Starter Kit II (Roche).

Conjugation assays

Conjugation assays from AN54 to recipient strains *Escherichia coli* C600 (rifampicin resistant) and *Escherichia coli* DH5α (nalidixic acid resistant) were performed. Mueller–Hinton agar plates (BD Bioxon) supplemented with rifampicin (100 μg/mL) or nalidixic acid (32 μg/mL) containing AN (32 μg/mL) and MEM (8 μg/mL) were used for the selection of transconjugant strains.

Nucleotide sequence accession numbers. Draft genome and plasmid sequences of the AN54 strain were deposited in the GenBank database, with accession numbers CP041224.1 to CP041229.1.

Results

In this work, we report the identification of an *Acinetobacter haemolyticus* (AN54) strain resistant to carbapenems, which was isolated from peritoneal dialysis fluid. This strain was initially identified as *Acinetobacter* spp. by a VITEK 2 System and subsequently reclassified as *A. haemolyticus* by analysis of the *rpoB* gene. The AN54 strain exhibited resistance to AN, and to the broad-spectrum β-lactams antibiotics, except for TIM. The bla_{NDM-1} and $bla_{OXA-265}$ were previously detected by PCR and sequencing. A twofold decrease in the MIC for AN and no change in the MICs for CTX and MEM in the presence of EPI were observed. In addition, the phenotypic test to detect MBL production was positive (Table 1).

Plasmid DNA extraction revealed that the AN54 isolate carries six different bands. Southern blot hybridization indicated that bla_{NDM-1} was present in one of the plasmids (Fig. 1).

Whole-genome sequencing analysis revealed that AN54 possess a chromosome with a size of ~3.61 Mbp, and we obtained the complete sequence of four plasmids named

TABLE 1. ANTIMICROBIAL SUSCEPTIBILITIES TEST AND GENOTYPE OF *ACINETOBACTER HAEMOLYTICUS* AN54

			MIC (μg/mL)							
Strain	*Anatomical site*	*Resistance phenotype*	*CAZ*	*FEP*	*IPM*	*CTX (CTX + EPI)*	*MEM (MEM + EPI)*	*AN (AN + EPI)*	*Genotype*[a]	*Efflux pumps genes*[a]
AN54	Peritoneal dialysis fluid	PIP, TIC, SAM, TZP, CAZ, FEP, CTX, CRO, IPM, MEM, AN	>128	>128	>128	>128 (>128)	>128 (>128)	64 (32)	bla_{NDM-1}, $bla_{OXA-265}$, *aph*A6, *aac*-(6')-Ig	*adeA*, *adeB*, *adeC*, *adeI*, *adeK*, *adeS*,[b] *adeR*,[b] *macA*, *macB*

Control strain *Acinetobacter haemolyticus* HNP11 MIC values: CTX: 16 μg/mL, CTX + EPI: 8 μg/mL; AN: 64 μg/mL, AN + EPI: 32 μg/mL.
[a]Obtained by PCR and whole-genome sequencing analysis.
[b]Regulators of AdeABC efflux pump.
AN, amikacin; CAZ, ceftazidime; CRO, ceftriaxone; CTX, cefotaxime; EPI, efflux pumps inhibitor (phenylalanine-arginine-β-naphthylamide) (PAβN) 25 mg/L; FEP, cefepime; IPM, imipenem; MEM, meropenem; MIC, minimal inhibitory concentration; PIP, piperacillin; SAM, ampicillin/sulbactam; TIC, ticarcillin; TZP, piperacillin/tazobactam.

FIG. 1. **(A)** PFGE-S1 gel showing the plasmids of *Acinetobacter haemolyticus* AN54 strain, Line 1: Control strain *Escherichia coli* NCTC 50192, Line 2: AN54. **(B)** Hybridization autoradiography with the bla_{NDM-1} probe (DIG-High Prime DNA Labeling and Detection Starter Kit II, Roche). The *white arrow* indicates that bla_{NDM-1} was detected in the plasmid pAhaeAN54e. The plasmid sizes were determined by WGS. PFGE, pulsed-field gel electrophoresis. Color images are available online.

pAhaeAN54a (4.7 kbp), pAhaeAN54c (11.4 kbp), and pAhaeAN54d (12.8 kbp) that do not carry antimicrobial resistance genes, and pAhaeAN54e (45.46 kbp) that carries bla_{NDM-1} and *aphA6*. In the chromosome, we detected the presence of $bla_{OXA-265}$ and the aminoglycoside-modifying enzyme-encoding gene *aac(6')-Ig*. In addition, genes for efflux pumps and their regulators that mediate multidrug resistance were found:, *adeA*, *adeB*, *adeC*, *adeI*, *adeK*, *adeR*, *adeS*, *macA*, and *macB*, as well as heavy metal resistance genes *czcA* and *arsH*.

The bla_{NDM-1} was located in the 45.46 kbp plasmid, which we named pAhaeAN54e. This plasmid has a 41% guanine-cytosine content and 53 open reading frames. Sixteen of these genes are related to plasmid maintenance and transfer functions (plasmid backbone) and include the plasmid-partitioning genes *parA* and *parB*, and transfer genes *traA*, *traC*, and *traD*, as well as some genes of the type IV secretion system (T4SS). Twelve genes of this plasmid are located within the composite transposon carrying bla_{NDM-1}, and the remaining genes encode 25 hypothetical

proteins. Conjugation assays were performed to test the transferability of this plasmid; however, no transconjugants were obtained under the tested conditions.

Plasmid pAhaeAN54e has 99% nucleotide identity, with 100% coverage, to plasmids pNDM-BJ02 (accession no. JQ060896.1) and pNDM-BJ01 (accession no. JQ001791.1) of *A. lwoffii*.[28] Figure 2 shows the detected differences, especially the insertion of seven genes encoding hypothetical proteins along the T4SS gene cluster, near the *parA* and *traA* genes, and a deletion of a hypothetical protein upstream of *traC*. The pAhaeAN54e plasmid also has a truncated composite transposon that is similar to transposon Tn125. Both these transposons consist of the IS*Aba14* insertion sequence and the *aphA6*, $bla_{\text{NDM-1}}$, *ble*, and *trpF* genes, but in contrast to Tn125, the truncated transposon of strain AN54 has only one copy of the insertion sequence IS*Aba125*. The structure of the truncated transposon is similar to that found in plasmid pNDM-BJ02 and to the partially sequenced plasmids pABC7926 (accession no. JQ080305.2) and pNDM-69122 (accession no. LN611576.1) of *A. haemolyticus* (Fig. 3).[29,30]

Plasmid pAhaeAN54e contains an IS*91* family transposase, which is 330 bp larger than the *ins*E transposase gene carried on the pNDM-BJ02 and pNDM-BJ01 plasmids in *A. lwoffii*. In addition, pAhaeAN54e has an insertion of two genes encoding hypothetical proteins downstream of IS*91* and one more that is adjacent to the recombinase gene.

Interestingly, plasmid pAhaeAN54e has 99% identity with more than 73% of coverage with the *P. rettgeri* plasmid p06-1619-NDM (accession no. KX832928.1) reported in Mexico (Fig. 4).[31] These plasmids share 33 genes, including *traA*, *traC*, *traD*, and genes from the type IV secretion system. Plasmid p06-1619-NDM also has a Tn125-like element.

Discussion

A. baumannii is one of the principal etiological agents causing nosocomial infections in Mexico and the rest of the world.[32–34] In Mexican hospitals, there are a few reports of carbapenem-resistant *Acinetobacter* isolates that are not included in the *A. calcoaceticus-baumannii* complex,[17] possibly because the molecular typing of these species is not routinely implemented in Mexican hospitals. In this work, we studied the resistome of an *A. haemolyticus* strain resistant to carbapenems. The strain carries different resistance mechanisms, such as $bla_{\text{OXA-265}}$, which is a member of the $bla_{\text{OXA-214}}$-like family on the *A. haemolyticus* chromosome.[35] The strain also harbors aminoglycoside-modifying enzyme genes, such as *aac(6')-Ig*, which is responsible for AN resistance and belongs to the AAC(6')-I aminoglycoside N-acetyltransferase family reported in *Acinetobacter* spp.,[36,37] and *aphA6*, which confers resistance to AN and is associated with the presence of $bla_{\text{NDM-1}}$ in a plasmid from this strain, as has been reported in another study.[38]

Another antibiotic resistance mechanism studied in *A. baumannii* is the use of efflux pumps, mainly the RND family of efflux pumps, such as the AdeABC and AdeIJK systems, which are located on the bacterial chromosome. This family exhibits a wide substrate range that includes dyes, biocides, detergents, and antiseptics; however, its presence has been little studied in non-*baumannii* spp.[39] The overexpression of this system was shown to be responsible for decreasing susceptibility to a broad spectrum of antimicrobials, such as aminoglycosides, tetracyclines, erythromycin, chloramphenicol, trimethoprim, fluoroquinolones, some β-lactams, and ethidium bromide, and has recently also been associated with tigecycline.[40] The genes that encode the AdeABC efflux pump are organized in an operon (*adeABC*). There are two regulatory genes, *adeS* and *adeR*, and their products are closely related to proteins of the two-component regulatory system. These genes regulate efflux pump expression in response to cellular environment stimuli (antibiotics). This type of expression is called inductive.[41]

AdeIJK encoded by the *ade*IJK operon is the second RND efflux system described in *A. baumannii*,[42] which contributes to the resistance to β-lactams, such as TIC, cephalosporins, aztreonam, fluoroquinolones, tetracyclines, tigecycline, lincosamides, rifampicin, and chloramphenicol; however, aminoglycosides are not substrates for this pump.[43] The *Acinetobacter haemolyticus* AN54 strain contains homologues of the regulator proteins AdeS and AdeR and the efflux pump components AdeA, AdeB and AdeC with the *Acinetobacter baumannii* AYE strain, these proteins share an amino acid identity of 72%, 85%, 86%, 92% and 77% respectively, to the corresponding proteins. In addition, homologues of AdeI and AdeK proteins with 82% and 89% identity with *Acinetobacter baumannii* AYE were also found. However, the AdeJ subunit in strain AN54 was not located.

In this work, EPI was used to evaluate the role of the efflux pump in antibiotic resistance. The results obtained indicate that the efflux pump only participates in conferring resistance to AN, as shown by a twofold decrease in the MIC (from 64 to 32 μg/mL). The strain did not show a decrease in the MICs of CTX and MEM, possibly due to the presence of $bla_{\text{NDM-1}}$.[6]

The presence of $bla_{\text{NDM-1}}$ in a non-*baumannii* spp. of environmental origin such as *A. haemolyticus* is medically and epidemiologically relevant. This is especially true considering the lack of successful treatment for patients with some underlying diseases and the ease of $bla_{\text{NDM-1}}$ dissemination through complex recombination events mediated by insertion sequences, transposons, and plasmids, as reported in previous works.[31,44,45]

The *Acinetobacter haemolyticus* AN54 strain harbors $bla_{\text{NDM-1}}$ within a Tn125-like transposon in a plasmid highly similar to pNDM-BJ02 of *A. lwoffii*, which has been previously reported in China.[28] The backbone of the plasmids carrying $bla_{\text{NDM-1}}$ between *Acinetobacter* spp. is relatively conserved; however, one difference found in our plasmid is the presence of IS*91*. This gene is designated as IS*CR*, and one of its functions is the mobilization of additional sequences upstream of the transposase gene.[46,47] The second difference is the insertion of seven genes encoding hypothetical proteins in the putative conjugation region; the function of these proteins is yet unknown. Currently, there are no methodologies to characterize this kind of replicon, due to the absence of a typical Rep protein, such as the one presented by Enterobacteriaceae plasmids, indicating that these plasmids possess an uncharacterized replication system; therefore, additional studies are needed. No transconjugants were obtained under the tested conditions. However, we cannot exclude other alternative mechanisms of plasmid

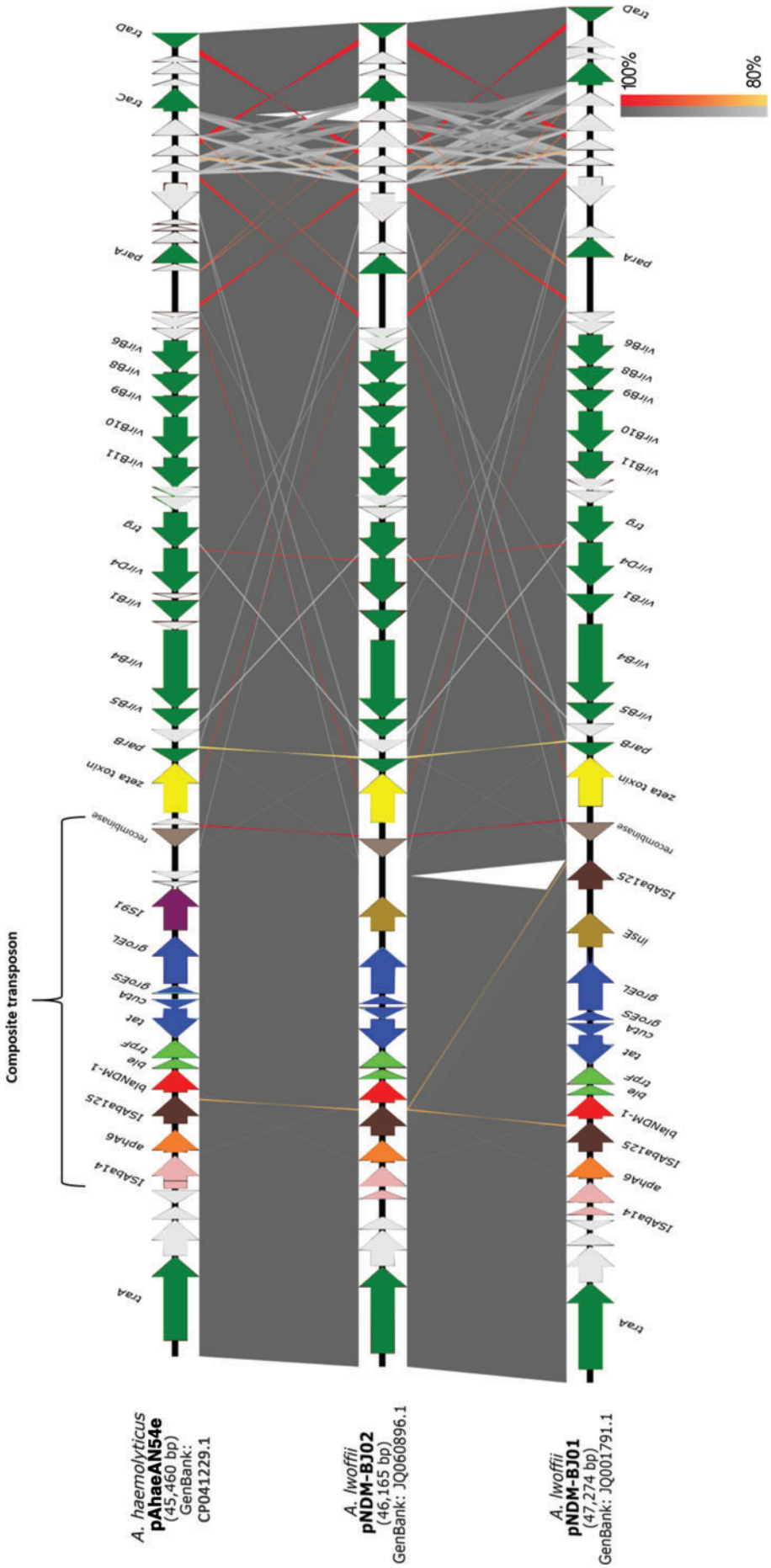

FIG. 2. The schematic representation and alignment of plasmid pAhaeAN54e of *Acinetobacter haemolyticus* and plasmids pNDM-BJ02 and pNDM-BJ01 of *Acinetobacter lwoffii* are divided into two sections: the composite transposon in which IS*Aba125*, bla_{NDM-1}, and IS*91* are inserted and the putative conjugation machinery. The known genes are marked in *green*, and putative proteins are marked in *white*. The *gray bars* indicate 99% identity, and the inverted regions are indicated in *red*. Color images are available online.

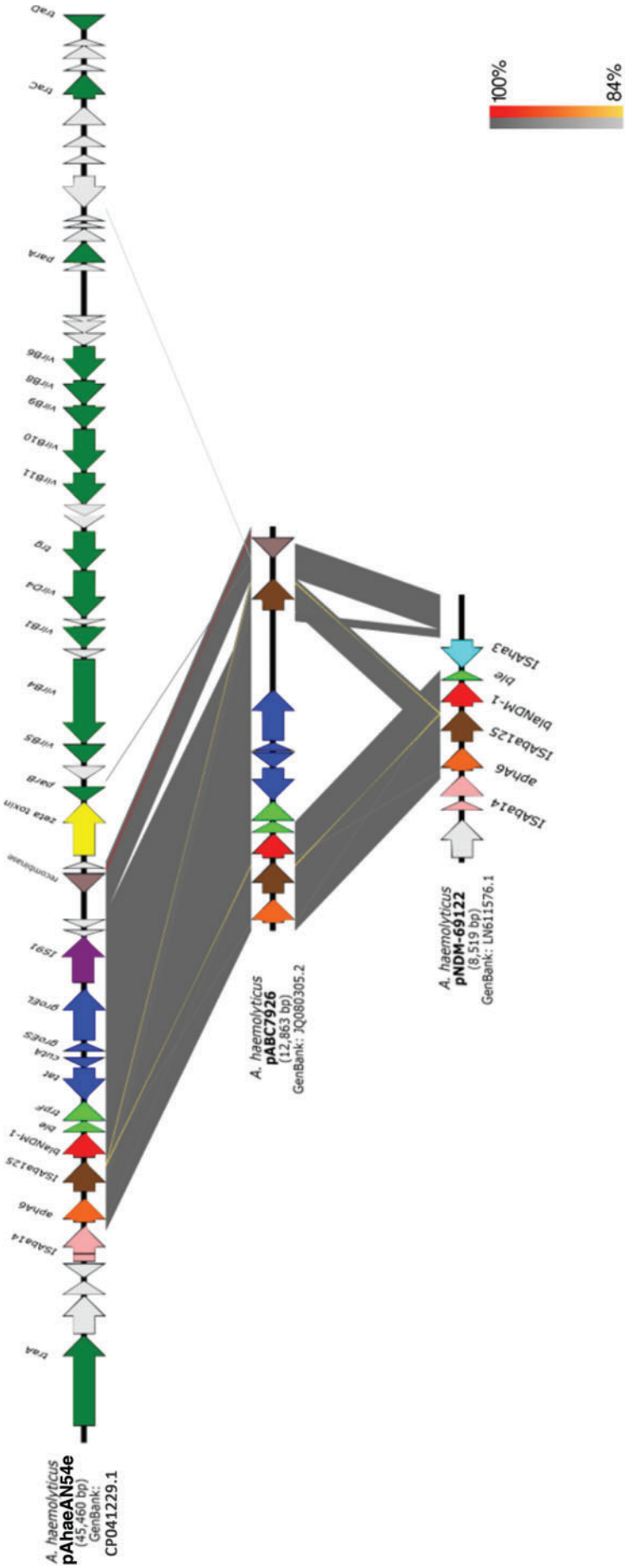

FIG. 3. Comparison between the plasmid pAhaeAN54e and the partially sequenced plasmids pABC7926 and pNDM69122 of *Acinetobacter haemolyticus*. Color images are available online.

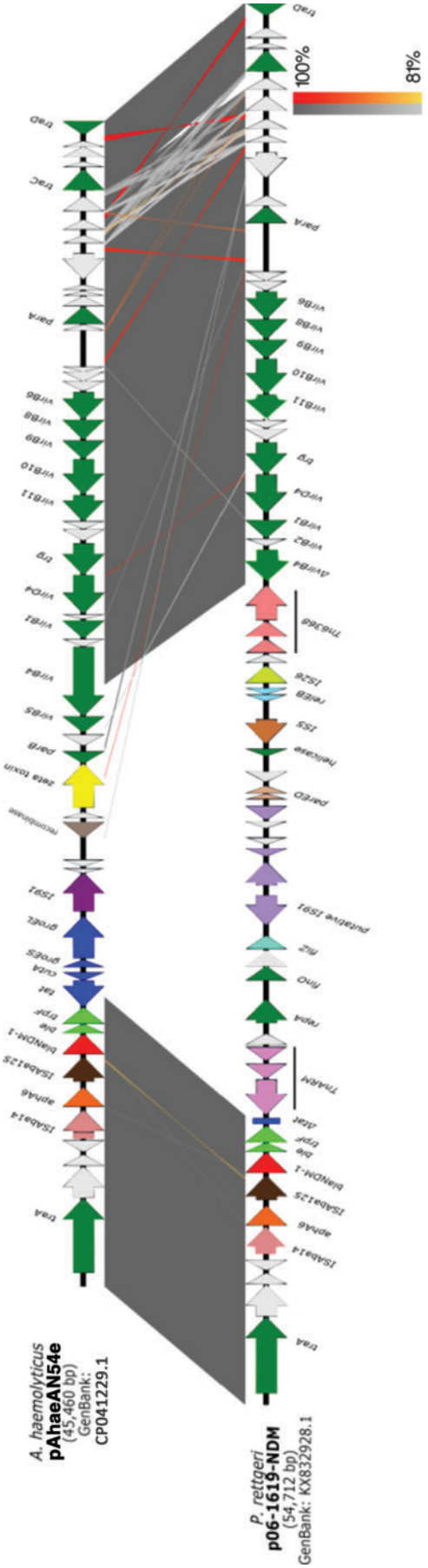

FIG. 4. Comparison between plasmids pAhaeAN54e of *Acinetobacter haemolyticus* and p06-1619-NDM of *Providencia rettgeri*, both of which are isolated from México. Color images are available online.

transference, as reported in other studies of *Acinetobacter* spp. carrying $bla_{\text{NDM-1}}$.[14,15,38,48]

Recently, Duran-Bedolla *et al.*[17] partially reported the genetic context of $bla_{\text{NDM-1}}$ in strains of *Acinetobacter* spp., including the *Acinetobacter haemolyticus* 10256 strain isolated in a different geographical area of Mexico. The plasmid reported in our work showed specific differences in comparison with the plasmid of *Acinetobacter haemolyticus* 10256 (55 kbp plasmid); these included size (45.4 kbp), absence of IS*Aba125* sequence in the 3′ end, presence of genes encoding hypothetical proteins along the structure of the plasmid, and IS*91* instead of IS*CR27*. These results suggest that the genetic context of $bla_{\text{NDM-1}}$ in *A. haemolyticus* is partially conserved.[17,29,30]

Our study shows that plasmid pAhaeAN54e has a high similarity with plasmid p06-1619-NDM of *P. rettgeri* and, as noted by Marquez-Ortiz *et al.*,[31] the similarities among pNDM-BJ01-like plasmids and plasmids of *P. rettgeri* may be the result of recombination events that lead to a chimeric plasmid that can be transmitted and replicated among Enterobacteriaceae and *Acinetobacter* isolates.[49]

Concluding Remarks

In this study, we report a multidrug-resistant clinical *A. haemolyticus* strain isolated from a pediatric patient carrying $bla_{\text{NDM-1}}$ in a novel variant of pNDM-BJ01-like plasmids. This plasmid could constitute a dissemination mechanism of antimicrobial resistance genes among diverse bacterial species. The findings reported in this work contribute to providing information about the diverse mechanisms of resistance that can coexist in *A. haemolyticus* strains.

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Ethical Statement

Acinetobacter haemolyticus AN54 isolate was collected during routine sampling, and patient data were anonymized. The protocol for this study was approved by the Ethical Committee of Hospital number: HNP/ENS/177/2016.

Disclosure Statement

No competing financial interests exist.

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Apéndice 5 - Unexplored Genetic Diversity of Multidrug- and Extremely Drug-Resistant *Acinetobacter baumannii* Isolates from Tertiary Hospitals in Honduras.

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Unexplored Genetic Diversity of Multidrug- and Extremely Drug-Resistant *Acinetobacter baumannii* Isolates from Tertiary Hospitals in Honduras

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Although *Acinetobacter baumannii* has become one of the most important nosocomial pathogens worldwide, very little is known about the genetic identity of isolates from less developed countries in Latin America. To alleviate this, we sequenced the genomes of 16 *A. baumannii* isolates from Honduras. Whole-genome sequencing was conducted on 16 isolates from five Honduran Hospitals. With the sequences of these Honduran isolates and other 42 publically available genomes, a maximum likelihood phylogeny was constructed to establish the relationship between the Honduran isolates and those belonging to the International Clones (ICs). In addition, sequence type (ST) assignation was conducted by the PubMLST, and antibiotic resistance genes were identified using ResFinder. The Honduran isolates are highly diverse and contain new allele combinations under the Bartual multilocus sequence typing scheme. The most common STs were ST_B447/ST_P10 and ST_B758/ST_P156. Furthermore, none of these isolates belongs to clonal complexes related to the ICs. Antibiotic susceptibility profiles of these isolates showed that they are multidrug resistant (MDR) or extensively drug resistant (XDR). In addition, the Honduran isolates had genes involved in resistance to seven antibiotic families. For instance, several *bla*OXA alleles were found, including *bla*OXA-23 and a gene encoding the metallo-beta-lactamase NDM-1. Notably, nine of the Honduran isolates have antibiotic resistance genes to three or more antibiotic families. In summary, in this study, we unveiled an untapped source of genetic diversity of MDR and XDR isolates; notably, these isolates did not belong to the well-known ICs.

Keywords: antibiotic resistance genes, blaOXA-23, NDM-1, International clonal complex

Introduction

Over the last decade, *Acinetobacter baumannii* has become one of the most prominent bacterial pathogens worldwide, as it has turned into one of the most problematic nosocomial pathogens in intensive care units in many parts of the world and a constant source of nosocomial infections, and a frequent cause of outbreaks. Actually, *A. baumannii* is at the very top of the 2017 World Health Organization Priority List for Research and Discovery of New Antibiotics. Although much information about *A. baumannii* clinical isolates has been gathered in developed countries, clinical isolates from many developing countries have received very little attention.

The limited amount of information available about *A. baumannii* isolates in Latin America shows that, although the International Clones (ICs) are present in this region of the world, they are not the most predominant.[1–3] For instance, even though Mexico shares a large border with the United States of America, the most common sequence type (ST) in Mexico is ST758, which belongs to clonal complex 636 (CC636, previously CC113) and is not related to CC92 (IC-II), the most prevalent in the United States of America.[4] Although some of us recently started to analyze the genomic diversity of *A. baumannii* isolates in Mexico,[5–7] the situation in Central America is worst and there is hardly any information about isolates for this region. To mitigate this lack of information, we carried out genome sequencing of 16 *A.*

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baumannii isolates sampled from five different hospitals in Honduras located in two different departments. Multilocus sequence typing (MLST) analysis indicated that five isolates had new allele combinations and none of the isolates was related to the ICs. Moreover, a phylogenomic analysis showed that the isolates were highly diverse and spread over the entire phylogenetic tree. In addition, most of the *A. baumannii* isolates possessed a wide variety of antibiotic resistance genes, including several *blaOXA* alleles and *bla*NDM1, a metallo-beta-lactamase capable of conferring resistance to a wide variety of beta-lactam antibiotics, including carbapenems. Our study shows that Honduras is an untapped source of genetic diversity of multidrug-resistant and extremely drug-resistant isolates of *A. baumannii.*

Methods

Isolate collection

Clinical isolates were obtained from patients with *A. baumannii* infections in five tertiary care hospitals located in two Honduran departments: Cortés and Francisco Morazán. Initial identification of isolates was performed using VITEK 2 (bioMérieux, France). Age, sex, and source of the samples are listed in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/mdr). Antimicrobial susceptibility profiles for all strains were determined using BD EpiCenter™ Microbiology Data Management System V7.22A (BD Diagnostics, France) at Department of Microbiology, Instituto Nacional de Cancerología (Supplementary Table S2). Multidrug resistant (MDR) and extensively drug resistant (XDR) were defined according to the criteria used by Magiorakos *et al.*[8]

Plasmid profiles

Plasmid profiles of the isolates were visualized on agarose gels according to the protocol described by Hynes and McGregor.[9] Briefly, cell were harvested in mid-logarithmic phase and lysed in the agarose gel well with lysozyme and sodium dodecyl sulfate before electrophoresis. Images were obtained staining the gel with ethidium bromide and photographed in a U.V. transilluminator.

Genome sequencing

DNA was obtained with the Genomic DNA Purification Kit (Thermo Scientific), using the manufacturer's instructions. Sequencing was performed at the Unidad de Secuenciación e Identificación de Polimorfismos, Instituto Nacional de Medicina Genómica (INMEGEN, México). DNA libraries were constructed with a TruSeq DNA Library Preparation Kit v3 and sequenced on an Illumina MiSeq platform with 2×300 bp paired-end reads. Genome assemblies were carried out using ABySS 2.0.1[10] and VELVET 1.2.10,[11] and the best assembly of each genome was deposited in GenBank and annotated with the NCBI annotation pipeline. The ST assignation of each *A. baumannii* isolate was obtained from the PubMLST database (https://pubmlst.org). Supplementary Table S3 gives the accession numbers and ST assignation of these isolates. eBURST analysis was performed with goeBURST[12] and the identification of acquired antibiotic resistance genes was done with ResFinder.[13]

Selection of homologous groups, model selection, and phylogenetic inferences

For the phylogenetic analysis, we used the genome assemblies of our *A. baumannii* collection and 41 genomes from NCBI (Supplementary Table S4). To obtain the homologous groups, we first blasted the proteome of each genome against the rest of the proteomes with a cutoff e-value of $1.0e-30$. Homologous protein groups were identified with PanOCT,[14] requiring that proteins within a homologous group show an aligned region of ≥90% of their lengths and ≥80% identity. For our phylogenetic analysis, we only considered single gene families (SGFs), which have only one gene per genome, and these were aligned with MUSCLE,[15] specifying 50 iterations. To create a DNA alignment in frame, we used the program TRANALING,[16] and to detect and discard SGFs with recombination signals, we used PhiPack[17] setting a *p*-value cutoff of 0.05. Then, the SGFs that did not show recombination signals were concatenated to form a superalignment. On this alignment, we constructed a maximum likelihood (ML) phylogeny using the generalized time-reversible model (GTR) gamma distributed rate variation among sites (+G) model, which was the most adequate model according to the jModelTest 2 program.[18] We ran a nonparametric bootstrap analysis (100 replicates) on the ML phylogeny to establish the support for the clades. To determine the relatedness between the Honduran strains, the number of single-nucleotide polymorphisms (SNPs) was determined from the concatenated alignment in a pairwise manner by MEGA 7.[19]

Results

The collection

Previous studies have suggested that the worldwide increase in *A. baumannii* infections is the result of the rapid expansion of a limited number of clones. The prevalence of the ICs and specifically, IC-II in Europe, North America, the Middle East, Asia, and Oceania suggests that this situation could also occur in the rest of the world. To explore which clones are circulating in Honduras and which antibiotic resistance genes they carry, we gathered a small collection of nosocomial isolates (16 isolates in total), from March 2015 to June 2016, sampled from five tertiary care health institutions located in two departments in Honduras. It is important to note that our collection was made without any specific criteria regarding the antibiotic resistance profiles of the isolates. The isolates were obtained from several patients and all were initially identified as *A. baumannii* as per the automated system VITEK 2. Antimicrobial susceptibility profiles revealed that 75% of the strains were XDR and the rest were MDR. All isolates were nonsusceptible to ampicillin, cefazolin, cefoxitin, ertapenem, and fosfomycin c/G6P. Half of the isolates were nonsusceptible to imipenem and 56.25% to meropenem. However, all were susceptible to colistin (Supplementary Table S2).

The isolates came from different sources (Supplementary Table S1); where the most abundant source was endotracheal tubes (36.4%), followed by blood (18.1%) and wounds (13.6%). To avoid working with the same or very closely related clones, the plasmid profiles of all strains were obtained by *in situ* lysis gel electrophoresis (Supplementary Fig. S1). This analysis showed that with the exception of

two isolates (read below), which had the same profile, the plasmid profiles of the isolates varied widely in terms of the number and size of plasmids. Therefore, almost each isolate possessed a distinctive plasmid profile, suggesting that, in general, they did not come from the same nosocomial outbreak. The exceptions were HEU2 and MCR54, collected in different hospitals located in distinct Honduran departments.

Phylogenetic relationships of the isolates

Then, to evaluate the relationships between the Honduran isolates and isolates obtained from other parts of the world, we sequenced the genome of all members of our collection with an Illumina MiSeq platform. We first used the genome sequences to define their STs under the MLST Bartual scheme.[20] We found that five isolates have new allele combinations and, in consequence, curators of the *A. baumannii* database (PubMLST) provided us with new ST numbers (Supplementary Table S2). Unfortunately, we did not obtain enough sequence information for isolate MCR6739 to establish its ST. In total, nine STs were found in these isolates and the most common were ST_B447 and ST_B758, each one with three isolates. It is important to point out that isolates from the ST_B447 have been recorded in the Czech Republic, United States of America, and Canada, whereas isolates belonging to ST_B758 have been described in Mexico and in Canada.[7,21] Another isolate (MCR10179) belongs to ST_B229, an ST that has been reported in Mexico, Brazil, and the United States of America (PubMLST database). Furthermore, the eBURST analysis showed that the Honduran isolates belong to nine different clonal complexes, none of them related with the clonal complexes embracing the ICs. The most common STs, ST_B447 and ST_B758, belonged to clonal complexes CC447 and CC636, respectively. These observations suggest the existence of new ICs preferentially circulating in North and South America. On the other hand, our collection also contains five isolates with new allele combinations, suggesting that they could be Honduran endemic clones not previously described. We also constructed a phylogenetic tree to establish more accurately the relationships between these isolates. The ML phylogenetic tree (Fig. 1) showed that the Honduran isolates are highly diverse as they are located, forming small clusters, on different parts of the tree. In line with this, we found that the average difference between any two Honduran strains was 1,456 SNPs. Furthermore, from this tree, it is clear that the Honduran isolates do not have a close association with isolates from the ICs. These results are congruent with eBURST analysis, considering that Honduran isolates sharing the same ST were located within the same clade. It is important to point out that despite the small sample studied here, these results indicate an unexpected diversity of *A. baumannii* in Honduras.

Antibiotic resistance genes

The second major goal of our study was to identify the antibiotic resistance genes present in the Honduran isolates and for that, we analyzed the draft genomes using the ResFinder database. A summary of these results is described in Table 1. The most common resistance genes were those related with beta-lactam antibiotics. As expected, all the isolates from our collection have two intrinsic encoded beta-lactamases: *bla*ADC-25 and *bla*OXA-51-like alleles (*bla*OXA-64, *bla*OXA-65, *bla*OXA-68, *bla*OXA-69, *bla*OXA-132, or *bla*OXA-180). In two isolates, the *bla*ADC-25 gene is closely associated with an ISAba1 element (HEU2 and MCR54) and in other with an ISAba27 element (MCR26739). To the best of our knowledge, an association between ISAba27 and a *blaA*DC allele has not been reported before. Four isolates HEU55808, HEU5, MCR6056, and MCR10179, obtained from two hospitals, contain a chromosomal encoded *bla*OXA-23 gene in close proximity with an ISAba1 element, suggesting that this element influences *bla*OXA-23 expression and potentially increases the minimal inhibitory concentrations for carbapenems.

Two different isolates obtained from the same hospital possess a *bla*NDM-1, a gene encoding a class B metallo-lactamase (MCR9238 and MCR10172, see Table 1). This gene is usually carried in plasmids; however, considering that we have only draft genomes of these isolates, it was not possible to determine whether *bla*NDM-1 is located on a plasmid; nonetheless, in both cases, this gene is linked to a Tn125-like element. We also found three isolates (HEU2, MCR54, and IHSS3526) harboring a *bla*CTX-M15 gene that encodes an extended-spectrum beta-lactamase, and in the HEU2 isolate, a carbenicillin-hydrolyzing beta-lactamase allele (*bla*CARB-8) was found; notably, to the best of our knowledge, this has not previously been reported in *A. baumannii*. Finally, we identified in isolate MCR6739 a gene linked to sulbactam resistance (*bla*TEM-1B).

In addition, we identified several genes related to aminoglycoside resistance and the most common were the *strAB* genes, which are involved in streptomycin resistance; these genes were found in 5 of the 16 isolates studied here. Another gene involved in aminoglycoside resistance was *arm*A, a 16S-methyltransferase gene that confers resistance to 4,6-disubstituted deoxystreptamines. This gene was found in isolates HEU2 and MCR54. Even more, we found three types of aminoglycoside modifying enzymes: first, *aad*A1 and *aad*A2 both encoding aminoglycoside adenyltransferases involved in streptomycin and spectinomycin resistance. Second, two genes encoding *O*-phosphotransferases: *aph(3′)-Ic* and *aph(3′)-Via*. Both genes linked to kanamycin, neomycin, and puromycin resistance, and the last one also to amikacin and gentamicin B resistance. Finally, three genes encoding *N*-acetyltransferases, involved in different types of aminoglycoside resistance, were found: *aac(3)-Ia* (gentamicin resistance), *aac(3)-IId* (gentamicin and tobramycin resistance), and *aac(3)-IIa.*

Besides the genes involved in resistance to beta-lactam and aminoglycoside antibiotics, we identified genes linked to resistance to other antibiotic families: in three isolates, we found the macrolide resistance genes *msrE* and *mphE.* Seven isolates possess a *sul2* gene and three possess a *sul1* gene, both related to sulfonamide resistance. Four isolates have genes related to tetracycline resistance; two possess a *tetB* gene and the others two possess *tetA* or *tetG*. A couple of isolates have a chloramphenicol resistance-related gene, *catA1*, and one of these also has a florphenicol resistance gene (*floR*). Finally, in three isolates, we identified genes involved in trimethoprim resistance: *dfrA1* in one isolate and *dfrA12* in the other two.

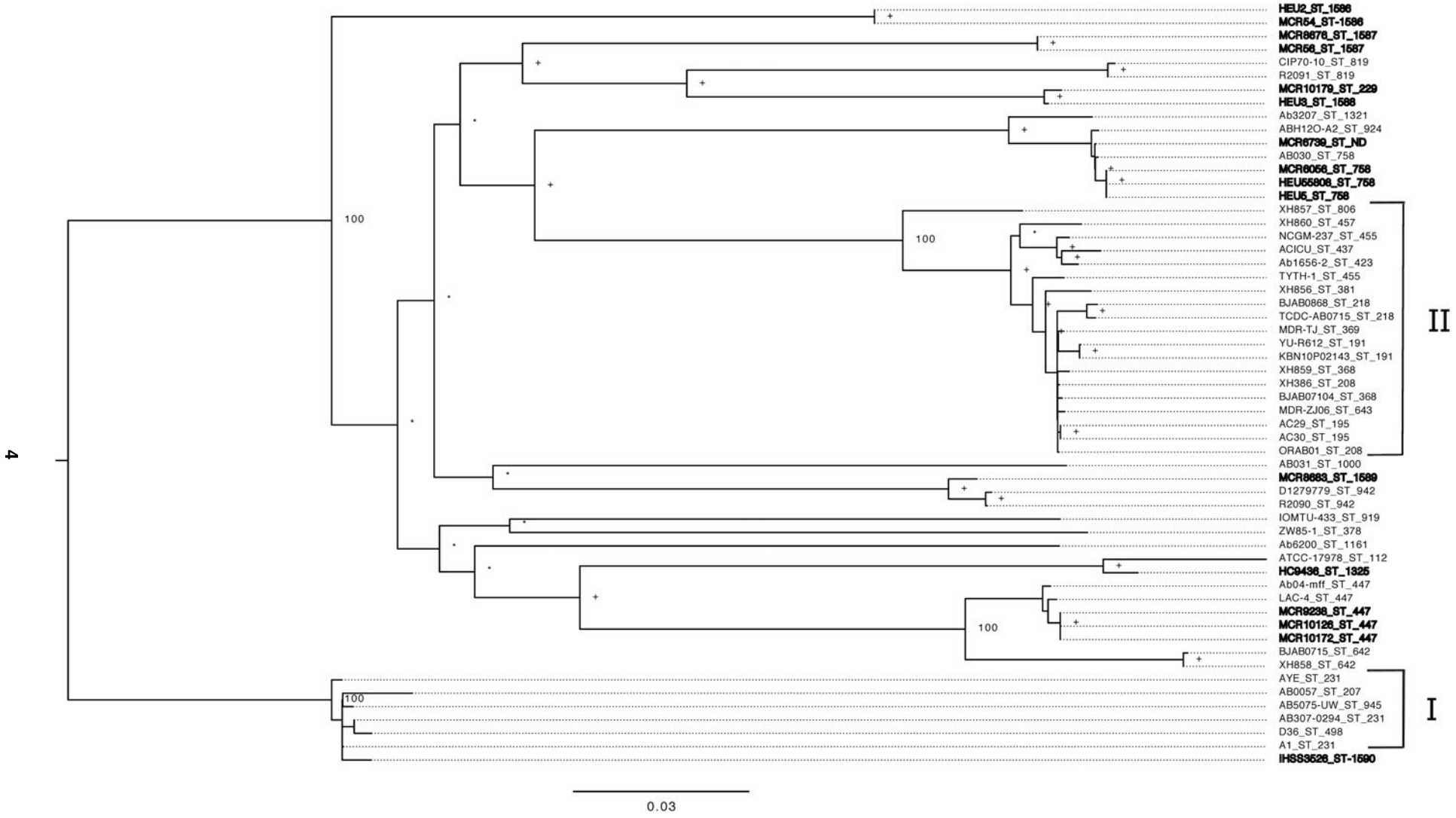

FIG. 1. Maximum likelihood phylogenetic tree. The tree was constructed on the concatenated alignment of all the single gene families not showing recombination signals. For each isolate the sequence type (Bartual scheme) is shown next to its name. Honduran isolates are indicated in *bold letters*. Isolates belonging to the International Complex II and I are marked with the two big *brackets*. For sake of clarity, the bootstrap values are shown just for some nodes; the rest of the nodes are marked with *plus signs* for those nodes with 70 or higher bootstrap support values, whereas *asterisks* denote nodes with <70 bootstrap support.

Table 1. Antibiotic Resistance Gene Profiles Based on the ResFinder Results

Isolate	*Aminoglycoside*	*Beta-lactam*	*Macrolide*	*Phenicol*	*Sulfonamide*	*Tetracycline*	*Trimethoprim*
HEU2	*aadA2* *aac(3)-IId* *armA*	*bla*CTX-M-15 *bla*ADC-25 *bla*OXA-132 *bla*CARB-8	*msrE* *mphE*	—	*sul*1 *sul*2	—	*dfr*A12
MCR56		*bla*ADC-25 *bla*OXA-65	—	—	—	—	—
MCR54	aadA2 *aac*(3)-IId armA	*bla*OXA-132 *bla*CTX-M-15 *bla*ADC-25	*msrE* *mphE*	—	*sul*1	—	*dfr*A12
MCR6739	*aac(3)-IId* *strAB*	*bla*TEM-1B *bla*OXA-65 *bla*ADC-25	—	—	*sul*2	—	—
HC9436		*bla*OXA-180 *bla*ADC-25	—	—	—	—	—
MCR6056	*strAB*	*bla*OXA-65 *bla*OXA-23 *bla*ADC-25	—	—	*sul*2	—	—
MCR8676		*bla*ADC-25 *bla*OXA-65	—	—	—	—	—
MCR8683	*aadA1* *aph(3′)-Ic*	*bla*ADC-25 *bla*OXA-180	—	*cat*A1 *flo*R	*sul*2	*tet*G	*dfr*A1
MCR9238	*strAB* *aac(3)-IIa*	*bla*OXA-68 *bla*ADC-25 *bla*OXA-180 *bla*NDM-1	—	—	*sul*2	*tetB*	—
MCR10126		*bla*ADC-25 *bla*OXA-68	*msrE* *mphE*	—	—	—	—
MCR10172		*bla*ADC-25 *bla*OXA-68 *bla*NDM-1	—	—	—	—	—
MCR10179		*bla*ADC-25 *bla*OXA-23 *bla*OXA-64	—	—	—	*tetB*	—
IHSS3526	*aadA1* *aac(3)-Ia* *aph(3′)-Via*	*bla*OXA-69 *bla*ADC-25 *bla*CTX-M-15	—	*cat*A1	*sul*1	*tetA*	—
HEU55808	*strAB*	*bla*OXA-65 *bla*ADC-25 *bla*OXA-23	—	—	*sul*2	—	—
HEU3		*bla*OXA-64 *bla*ADC-25	—	—		—	—
HEU5	*strAB*	*bla*ADC-25 *bla*OXA-23 *bla*OXA-65	—	—	*sul*2	—	—

Discussion

Given that the Honduran isolates were scattered over the phylogeny, the considerable number of STs for just 16 isolates and the fact that almost every Honduran isolate had its own plasmid profile, we conclude that the Honduran isolates are highly diverse and that they might have had multiple origins.

These observations suggest the existence of potential new ICs preferentially circulating in North and South America. Furthermore, the five isolates with new allele combinations could be Honduran endemic clones, but more data are needed to corroborate this, nonetheless. Importantly, all isolates studied here were MDR and even XDR, and contain a wide variety of acquired antibiotic-resistant genes; a worrisome situation for the clinicians and infection control teams of the hospitals sampled—if one considers that these isolates were collected regardless of antibiotic resistance profiles. Furthermore, this also highlights the urgent need to reinforce all measures required to detect, destroy, and prevent the further spread of these lineages. On that account, these Honduran isolates not only possess a high genetic diversity but also show MDR and XDR phenotypes.

In summary, these Honduran isolates show an untapped source of genetic diversity of this pathogen and, worryingly, some of them are MDR and XDR. All in all, this study highlights the urgent need to study isolates of this pathogen from other understudied geographic areas, as there may yet be a considerable amount of unexplored genetic diversity.

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No competing financial interests exist.

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Apéndice 6 - Whole-Genome Sequences of Five A*cinetobacter baumannii* Strains from a Child with Leukemia M2.

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Whole-Genome Sequences of Five *Acinetobacter baumannii* Strains From a Child With Leukemia M2

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Acinetobacter baumannii is an opportunistic pathogen and is one of the primary etiological agents of healthcare-associated infections (HAIs). *A. baumannii* infections are difficult to treat due to the intrinsic and acquired antibiotic resistance of strains of this bacterium, which frequently limits therapeutic options. In this study, five *A. baumannii* strains (810CP, 433H, 434H, 483H, and A-2), all of which were isolated from a child with leukemia M2, were characterized through antibiotic susceptibility profiling, the detection of genes encoding carbapenem hydrolyzing oxacillinases, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), adherence and invasion assays toward the A549 cell line, and the whole-genome sequence (WGS). The five strains showed Multidrug resistant (MDR) profiles and amplification of the bla_{OXA-23} gene, belonging to ST758 and grouped into two PFGE clusters. WGS of 810CP revealed the presence of a circular chromosome and two small plasmids, pAba810CPa and pAba810CPb. Both plasmids carried genes encoding the Sp1TA system, although resistance genes were not identified. A gene-by-gene comparison analysis was performed among the *A. baumannii* strains isolated in this study and others *A. baumannii* ST758 strains (HIMFG and INCan), showing that 86% of genes were present in all analyzed strains. Interestingly, the 433H, 434H, and 483H strains varied by 8–10 single-nucleotide variants (SNVs), while the A2 and 810CP strains varied by 46 SNVs. Subsequently, an analysis using BacWGSTdb showed that all of our strains had the same resistance genes and were ST758. However, some variations were observed in relation to virulence genes, mainly in the 810CP strain. The genes involved in the synthesis of hepta-acylated lipooligosaccharides, the *pgaABCD* locus encoding poly-β-1-6-*N*-acetylglucosamine, the *ompA* gene, Csu pili, *bap*, the two-component system *bfms/bfmR*, a member of the phospholipase D family, and two iron-uptake systems were

identified in our *A. baumannii* strains genome. The five *A. baumannii* strains isolated from the child were genetically different and showed important characteristics that promote survival in a hospital environment. The elucidation of their genomic sequences provides important information for understanding their epidemiology, antibiotic resistance, and putative virulence factors.

Keywords: *Acinetobacter baumannii*, resistance, molecular typing, adherence, invasion, virulence factors, whole-genome sequence analysis

INTRODUCTION

Acinetobacter baumannii is an emerging opportunistic pathogen involved in healthcare-associated infections (HAIs) with elevated morbidity and mortality, particularly in immunocompromised patients. *A. baumannii* primarily causes ventilator-associated pneumonia and wound and burn infections, but is also an important cause of urinary tract infections and nosocomial septicemia (Gaynes and Edwards, 2005; Dijkshoorn et al., 2007). Treatment for *A. baumannii* infections is complex due to the increasing antibiotic resistance of this pathogen, which involves several intrinsic and acquired resistance mechanisms, such as the production of β-lactamase inhibitors and low-permeability outer membrane and efflux pumps (Peleg et al., 2008). The primary concern regarding HAIs-related *A. baumannii* strains is their high resistance to antibiotic therapy and the appearance of new strains that are resistant to all clinically available antibiotics (Peleg et al., 2008).

The study of the molecular epidemiology of bacterial pathogens is an essential tool for establishing control measures for hospital infections, such as the elimination or prevention of the further spread of *A. baumannii* strains inside a hospital. Diverse molecular typing methods have been used for epidemiological characterization of HAIs pathogens, including *A. baumannii* strains. Pulsed-field gel electrophoresis (PFGE) is a widely used method of choice to discriminate bacterial strains from nosocomial outbreaks (Urwin and Maiden, 2003). Multilocus sequence typing (MLST) is used to study population structures of bacterial pathogens. Several studies have showed typified *A. baumannii* strains using two MLST methods: the Oxford and Pasteur schemes (Bartual et al., 2005; Diancourt et al., 2010).

A study of *A. baumannii* of several outbreaks from different countries using the Oxford scheme and PFGE analysis identified a sequence type (ST) with the same subdivision, whereas the Pasteur scheme did not identify differences between outbreaks. Additionally, a major resolution of different outbreaks, including the identification of *gpi* and *gyrB* genes, has been described with the Oxford scheme, although this method is still less discriminative than the PFGE test (Tomaschek et al., 2016). Whole-genome sequencing (WGS) allows putative virulence factors of clinical bacterial strains to be identified (Beres et al., 2010), and in the case of hospital outbreaks, WGS allows colonized patients to be identified and to distinguish the possible transmission route of bacterial populations (Reuter et al., 2013). However, the discrimination criteria for clinical strains from outbreaks and non-outbreaks, as well as between clonal lineages, is not always clearly defined using WGS, and epidemiological data are still required (Leekitcharoenphon et al., 2014). However, the number of SNVs observed between isolates in a temporal frame may bring attention to a putative outbreak (Eyre et al., 2013; Inns et al., 2017). In contrast, the criteria used to establish and discriminate bacteria involved in hospital and nonhospital outbreaks have been well described using PFGE, Rep-polymerase chain reaction (PCR), and MLST tests (Fitzpatrick et al., 2016; Willems et al., 2016).

Comparative genomic analyses of some hypervirulent *A. baumannii* strains have allowed for genomic regions to be identified that contribute to the acquisition of antibiotic resistance, the establishment of colonization and invasion, and ST classification without the MLST analysis requirement (Ou et al., 2015; Zhang et al., 2018). The ST of clinical strains provides relevant information regarding the origin of clonal complexes, including their population distribution, which is epidemiologically important (Higgins et al., 2012). The goal of this study was to compare five *A. baumannii* strains isolated from a child with leukemia M2 using classical molecular typing (PFGE and MLST) and WGS using Illumina and PacBio platforms.

MATERIALS AND METHODS

Identification of *A. baumannii* Strains

The *A. baumannii* strains were cultured on Brucella blood agar from BD Difco (Madrid, Spain) and phenotypically identified at the Laboratorio Clínico Central of HIMFG using a Vitek® 2 automated system (BioMérieux, Marcy l'Étoile, France).

Antibiotic Susceptibility Tests

Antibiotic susceptibility testing was performed using the broth microdilution method. The antibiotics evaluated included the following: piperacillin (penicillin); piperacillin-tazobactam (β-lactam combination agents); ceftazidime, and ceftriaxone (cephems); imipenem and meropenem (carbapenems); colistin (lipopeptide); gentamicin (aminoglycoside); ciprofloxacin and levofloxacin (fluoroquinolones); trimethoprim-sulfamethoxazole (folate pathway antagonists); and tigecycline (glycylcycline). *A. baumannii* ATCC®19606™ was used as a quality control strain, and classification was performed according to the Clinical Laboratory Standards Institute [CLSI], 2018. Susceptibility to tigecycline was interpreted according to the US Food and Drug Administration (FDA) breakpoints for Enterobacteriaceae.

Detection of *bla*$_{OXA-LIKE}$ Carbapenemase Genes

Genomic DNA was extracted from the *A. baumannii* strains using a Quick-DNA Universal kit (Zymo, Irvine, CA, United States), and the *bla*$_{OXA-LIKE}$ genes were amplified by PCR. The specific primers used for PCR are listed in **Table 1**. PCR assays were performed using the following thermocycling conditions: 94°C for 5 min; 30 cycles at 94°C for 25 s, 52°C for 40 s, and 72°C for 50 s; and a final cycle at 72°C for 6 min. The DNA products were separated by electrophoresis in 1.8% agarose gels and stained with 0.5 mg/mL ethidium bromide solution. The stained gels were visualized and analyzed under a transilluminator (Bio-Rad, San Francisco, CA, United States). *A. baumannii* ATCC®19606™ was used as a positive control for *bla*$_{OXA-51}$.

PFGE Assays

The *A. baumannii* strains were plated onto Brucella blood agar and incubated at 37°C for 18 h. Approximately 5–10 colonies were selected and resuspended in 1 mL of Negative Gram Suspension Buffer (NGSB) (100 mM Tris–HCl and 100 mM EDTA 100, pH 8). From this bacterial suspension, 400 μL was embedded into 1% agarose plugs (SeaKem, Cambrex, Rockland, MD, United States), lysed with 5 mL of lysis buffer at pH 8.0 [0.5 M Tris–HCl, 0.5 M EDTA, 1% *N*-lauryl sarcosine sodium salt, and 25 μL of proteinase K (20 mg/mL)] with constant stirring (200 rpm) and incubated overnight at 54 ± 2°C. Subsequently, the samples were washed (MilliQ water at 50°C and 6× TE buffer) and digested with the enzyme *Apa*I (Promega, Madison, WI, United States). The digested chromosomal DNA was electrophoresed on 1% agarose gels (Bio-Rad, Hercules, CA, United States) using the CHEF MAPPER system (Bio-Rad, Hercules, CA, United States) in 0.5× TBE (AMRESCO, United States) under the following conditions: initial time of 5.0 s, final time of 30.0 s, 6 V/cm, with an inclination angle of 120 and a running time of 24 h. A lambda marker (Biolabs, Hertfordshire, United Kingdom) was used as molecular weight marker. Subsequently, the gels were stained with 0.5 mg/mL of ethidium bromide for 40 min and visualized under UV light. The DNA fragment patterns generated by PFGE were analyzed and compared using NTSYS version 2.2 (Applied Biostatistics, Setauket, New York, NY, United States) with the unweighted pair group method using arithmetic averages (UPGMA) algorithm and the DICE correlation coefficient (Tenover et al., 1995).

MLST

Amplification of the seven housekeeping genes (OXFORD scheme) was performed according to the protocol proposed by Bartual et al. (2005) using the specific primers listed in **Table 1**.

Polymerase chain reactions contained 100 ng of genomic DNA from each strain for the five genes (*gltA*, *gyrB*, *recA*, *cpn60*, and

TABLE 1 | Specific primers used to amplify *bla*$_{OXA-LIKE}$ genes and MLST data.

Gene	Primer sequences 5'–3'	Amplified (bp)	PCR	Reference
bla$_{OXA-23}$	GATCGGATTGGAGAA CAGA	501	OXA	Hujer et al., 2006
	ATTTCTGACCGCATTTCCAT			
bla$_{OXA-24}$	GGTTAGTTGGCCCCCTTAAA	246	OXA	Hujer et al., 2006
	AGTTGACGCAAAAGGGGATT			
bla$_{OXA-51}$	TAATGCTTTGATCGGCCTTG	353	OXA	Hujer et al., 2006
	TGCATTGCACTTCATCTTGG			
bla$_{OXA-58}$	AAGTATTGGGGCTTGTGCTG	453	OXA	Hujer et al., 2006
	CCCCTCTGCGCTCTACATAC			
gltA F	AAT TTACAGTGGCACATTAGGTCCC	722	MLST	Bartual et al., 2005
gltA R	GCAGAGATACCAGCAGAGATACACG		Amp/Seq	
gyrB F	TGAAGG CGGCTTATCTGAGT	594	MLST	Bartual et al., 2005
gyrB R	GCTGGGTCTTTTTCCTGACA		Amp/Seq	
gdhB 1F	GCTACTTTTATGCAACAGAGCC	774	MLST Amp	Bartual et al., 2005
gdh secF	ACCATGCTTTGTTATG		Seq	
gdhB 775R	GTTGAGTTGGCGTATGTTGTGC	774	MLST Amp	Bartual et al., 2005
gdh secR	GTTGGCGTATGTTGTGC		Seq	
recA F	CCTGAATCTTCYGGTAAAAC	425	MLST	Bartual et al., 2005
recA R	GTTTCTGGGCTGCCAAACATTAC		Amp/Seq	
cpn60 F	GGTGCTCAACTTGTTCGTGA	640	MLST	Bartual et al., 2005
cpn60 R	CACCGAAACCAGGAGCTTTA		Amp/Seq	
gpi F	GAAATTTCCGGAGCTCACAA	456	MLST	Bartual et al., 2005
gpi R	TCA GGA GCA ATACCCCACTC		Amp/Seq	
rpoD F	ACC CGT GAA GGT GAA ATC AG	672	MLST	Bartual et al., 2005
rpoD R	TTC AGC TGG AGC TTT AGC AAT		Amp/Seq	

OXA-23 (bla$_{OXA-23}$*), OXA-24 (bla*$_{OXA-24}$*), OXA-51 (bla*$_{OXA-51}$*), and OXA-58 (bla*$_{OXA-58}$*). Citrate synthase (gltA), DNA gyrase subunit B (gyrB), glucose dehydrogenase B (gdhB), homologous recombination factor (recA), 60-kDa chaperonin (cpn60), glucose-6 phosphate isomerase (gpi); RNA polymerase sigma factor (rpoD). Amp, Amplification; Seq, sequencing.*

rpoD) and the following PCR thermocycling conditions were used: 94°C for 2 min; 35 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s; and a final cycle of 72°C for 5 min. For the *gdhB* and *gpi* genes, the following PCR thermocycling conditions were used: 94°C for 5 min; 35 cycles at 94°C for 1 min, 57°C for 1 min, and 72°C for 2 min; and a final cycle at 72°C for 7 min. The DNA amplicons were resolved in 1.0% agarose gels in TAE 1× buffer, stained with 0.5 mg/mL ethidium bromide, and visualized under a transilluminator.

Only the *gdhB* and *gpi* genes were cloned. The DNA products were ligated into the cloning vector pJet1.2/blunt (Thermo Fisher Scientific, Waltham, MA, United States). Subsequently, the ligation mixture was used to transform *E. coli* DH5α competent cells, and the resulting colonies carrying the genes cloned into the pJet1.2/blunt vector were verified by PCR assays. The transformed plasmid was extracted using a Plasmid Miniprep kit (Zymo, Irvine, CA, United States), purified, and sequenced by Sanger sequencing. The sequencing reaction was performed using specific primers corresponding to the housekeeping and the cloning vector pJET1.2/blunt using BigDye-Terminator v3.1 Cycle Sequencing and an automatic ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, United States). The obtained sequences for each gene were analyzed using the database for *A. baumannii* strains[1] and characterized by a pattern defining its ST.

Adherence Assays

The type II pneumocyte cell line A549, derived from human lung carcinoma (A549 ATCC®CCL85; Manassas, VA, United States), was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium from GIBCO (Thermo Scientific, Waltham, MA, United States) supplemented with 10% fetal bovine serum (FBS) from GIBCO (Thermo Scientific, Waltham, MA, United States). Briefly, cell monolayers at 70–80% confluence in 24-well plates containing 1 mL of RPMI 1640 medium were infected at a MOI of 100:1 and incubated at 37°C with 5% CO_2 for 4 h. The *A. baumannii* strains used to infect the A549 cells were cultured in Brain Heart Infusion (BHI) broth overnight at 37°C. The nonattached bacteria were removed and washed three times with 1× PBS. Subsequently, the bacteria attached to the cell monolayers were removed by adding 1 mL of 0.1% Triton X-100 (Amresco, Solon, OH, United States), and serial dilutions were plated onto BHI agar plates and incubated to determine the number of colony-forming units (CFU)/mL. The adherence assays were performed in triplicate on three different days, and the data are expressed as the mean of the averages.

Invasion Assays

The A549 cell monolayers were prepared and infected as previously described in the adherence assay section. The infected cell monolayers were washed with 1× PBS and incubated with RPMI 1640 medium supplemented with 300 μg/mL lysozyme (Sigma–Aldrich, St. Louis, MO, United States) and 300 μg/mL gentamicin (Sigma–Aldrich, St. Louis, MO, United States) for 2 h at 37°C with 5% CO_2. The infected cell monolayers were washed three times with 1× PBS, detached with 1 mL of 0.1% Triton X-100, and plated onto BHI agar plates. The invasion frequency was calculated as the number of surviving bacteria after treatment with gentamycin and lysozyme divided by the total number of quantified bacteria from the adherence assays (Arikawa and Nishikawa, 2010). The invasion assays were performed in triplicate on three different days, and the data are expressed as the mean of the averages.

Whole-Genome Sequence Analysis

DNA from the *A. baumannii* strains was obtained using a Puregene Yeast/Bact Kit B from Qiagen following the manufacturer's instructions. The complete genomic sequence of strain 810CP was generated using reads from one SMRT cell of a PacBio RSII platform. The subreads were assembled *de novo* using the RS hierarchical genome assembly process (HGAP) protocol version 3 in SMRT analysis version 2.3 (Pacific Biosciences). To improve regions of low coverage, a genomic DNA shotgun library was prepared using the standard Illumina TruSeq protocol. Sequencing was performed using Illumina Nextseq500 2 × 75 bp paired-end chemistry, and a hybrid assembly was constructed using Unicycler v0.4.1 (Wick et al., 2017). Contigs corresponding to the chromosome and plasmids in the hybrid assembly were circularized both with Unicycler and a Perl script (available at https://github.com/jfass/apc). Draft genomic sequences of strains 433H, 434H, 483H, and A2 were obtained using Illumina Nextseq500 2 × 75 bp, and the reads were assembled with SPAdes 3.11.0. The sequence statistics are shown in **Supplementary Table 1**. Functional annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline and Prokka (Seemann, 2014). Reads were realigned against the final assemblies with bwa-mem (Li and Durbin, 2009), and genomic coverage was calculated using bedtools genomecov (Quinlan, 2014). The acquired antibiotic-resistance genes were identified using ResFinder (Zankari et al., 2012) and the BacWGSTdb platform (Ruan and Feng, 2016). Insertion sequences (ISs) were identified with ISfinder[2] (Siguier et al., 2006). Prophage-related sequences were identified using PHAST[3] (Zhou et al., 2011). Genomic islands were predicted using IslandViewer 4 with the SIGI–HMM algorithm, which is part of this computational suit (Waack et al., 2006; Bertelli et al., 2017). Virulence-associated genes were identified using the virulence factor database (VFDB)[4] (Chen et al., 2005) and the BacWGSTdb platform (Ruan and Feng, 2016). Images of genome comparisons were generated using GenVision, a component of the DNASTAR Lasergene Core Suite. The complete genomes obtained in the present study were compared, and against other Mexican strains belonging to ST758 (Graña-Miraglia et al., 2017) using MUMmer 3.0 (Kurtz et al., 2004) and BLAST (Altschul et al., 1990). Gene content matrices were obtained using Roary (Page et al., 2015), with a minimum 90% identity between coding sequences (CDS) as a requisite for a gene to belong to the same family. Single-nucleotide variants (SNVs) between pairs of strains were assessed

[1]http://pubmlst.org/abaumannii/

[2]http://www-is.biotoul.fr

[3]http://phast.wishartlab.com

[4]http://www.mgc.ac.cn/VFs/main.htm

using MUMmer from unambiguous mappings. In addition, SNVs from monocopy core genes with no recombination signal detected by PhiPack (Bruen et al., 2006) were extracted using single nucleotide polymorphism (SNP) sites (Page et al., 2015). SNVs counts (omitting indels) and gene content matrices were plotted with ComplexHeatmap (Gu et al., 2016) in R 3.2.2.

Statistical Analyses

The data were analyzed using IBM SPSS version 19.0 (SPSS Inc., Chicago, IL, United States). Student's *t*-test was used to compare the adherence and invasion among the clinical strains and ATCC®19606™, and $p \leq 0.05$ was regarded as significant.

GenBank Accession Number

The GenBank accession numbers of the genome sequences obtained or used in this study are listed in **Supplementary Table 2**.

RESULTS

Clinical Description

The pediatric patient was previously healthy, presented at Hospital Infantil de México Federico Gómez (HIMFG) in December 2014 for pancytopenia and a mediastinal tumor, and by means of bone marrow aspirate, the patient was diagnosed with acute myeloid leukemia subtype M2. Two weeks after receiving the first cycle of chemotherapy, the patient was admitted to the emergency ward for a 24-h evaluation, which was characterized by a fever of 38°C, vomiting, colicky abdominal pain, and decreased stools. The patient did not improve with crystalloid administration and therefore required management with vasoactive drugs. Subsequently, the patient was diagnosed with septic shock, and antimicrobial therapy (meropenem, vancomycin, and amphotericin B) was initiated. The blood cultures and uroculture showed no development of microorganisms.

The pediatric patient entered intensive care for advanced management, persistent high-grade fever, and a systemic inflammatory response with persistence of profound neutropenia. Treatment with voriconazole was supplementary due to suspicion of pulmonary aspergillosis and without improvement in the thermal curve. On the eighth day of hospitalization, the patient presented hemodynamic deterioration with septic shock, which required orotracheal intubation and the reinitiation of vasoactive amines. However, the patient died in less than 24 h due to torpid evolution with septic shock refractory to amines, conditioned acute renal injury, disseminated intravascular coagulation, ventilator deterioration, and multiple organ failure. A total of five strains were obtained from this patient, the first strain was isolated on January 7, 2015 from stool (strain 810CP). Subsequently, three additional strains were isolated on January 11, 2015 from the bloodstream at different times (strains 433H, 434H, and 483H), and a final strain isolated on January 12, 2015 from cerebrospinal fluid (strain A-2) after performing an autopsy.

A. baumannii Strains Were Multidrug Resistant (MDR)

The MIC values for all *A. baumannii* strains exhibited resistance to the following six categories of antibiotics: penicillin, the β-lactam combination, cephem, carbapenems, fluoroquinolones, and the folate pathway antagonists. Only two strains were resistant to gentamycin (433H and A-2). Additionally, the *A. baumannii* strains showed a susceptibility profile to the lipopeptide and glycylcycline categories (**Table 2**).

A. baumannii Strains Harboring bla_{OXA-51} and bla_{OXA-23} Genes

To determine whether the five *A. baumannii* strains harbored genes related to carbapenem resistance, the presence of $bla_{OXA-LIKE}$ genes was assessed by PCR assays. From the five *A. baumannii* strains, a 353-bp product was amplified that corresponded to the bla_{OXA-51} gene encoding OXA-51, an intrinsic oxacillinase, and a 501-bp product corresponding to the bla_{OXA-23} gene encoding OXA-23, an oxacillinase associated with carbapenem resistance (data not shown). In addition, the genes encoding OXA-24 and OXA-48 were not identified in the five *A. baumannii* strains.

TABLE 2 | Resistance profiles of *A. baumannii* strains.

Clinical isolate	Antibiotics (MIC μg/mL)										
	PIP	TZP	CAZ	CRO	IPM	MEM	CL	GM	CIP	SXT	TGC
810CP	256	256/4	128	256	64	64	1	8	8	32/608	2
433H	256	256/4	128	256	32	64	1	16	8	32/608	2
434H	256	256/4	128	256	64	64	1	8	8	32/608	2
483H	256	256/4	128	256	64	64	1	8	8	32/608	2
A-2	256	256/4	128	256	32	32	1	16	8	32/608	2
ATCC®19606™	32	64/4	32	32	0.25	0.5	0.5	4	1	304/16	2
Cut-off	≥ 128	≥128/4	≥ 32	≥ 64	≥ 8	≥ 8	≥ 4	≥ 16	≥ 4	≥4/76	≥ 8
%R	100	100	100	100	100	100	0	40	100	100	0

*Piperacillin (PIP), piperacillin/tazobactam (TZP), ceftazidime (CAZ), ceftriaxone (CRO), imipenem (IPM), meropenem (MEM), colistin (CL), gentamicin (GM), ciprofloxacin (CIP), trimethoprim/sulfamethoxazole (SXT), tigecycline (TGC), *Cut-off values for resistance to MIC (μg/mL), percentage of resistant (%R).*

Clonal Relationship of the *A. baumannii* Strains

In this study, the *A. baumannii* strains obtained at different times from the same patient were evaluated for their clonal type based on PFGE pattern analyses. The five strains were grouped in two clusters, I and II; while, *A. baumannii* strain ATCC®19606™ was grouped as an independent cluster. These clusters displayed a macrorestriction pattern consisting of DNA fragments with molecular weights of 48.5 to 339.5 kb. In cluster I, *A. baumannii* strains A-2 and 810CP showed an identical macrorestriction pattern. In cluster II, *A. baumannii* strains 433H, 434H, and 483H also showed the same macrorestriction pattern, while the *A. baumannii* strain ATCC®19606™ was grouped into cluster III. A DICE correlation of 0.9993 and a 95% similarity index was determined in comparisons among the five *A. baumannii* strains. *Salmonella enterica* serotype Newport AM01144 was used as an external control (**Figure 1**).

Identification of STs in the *A. baumannii* Strains

Multilocus sequence typing sequences of the five *A. baumannii* strains belonged to ST758 with the following allelic profile: *cpn60* (28); *gdhB* (8); *gltA* (1); *gpi* (106); *gyrB* (17); *recA* (10); and *rpoD* (32). According to eBURST, ST758 belongs to clonal complex 636 and shares the same distribution cluster with the other highly frequent STs (**Figure 2**).

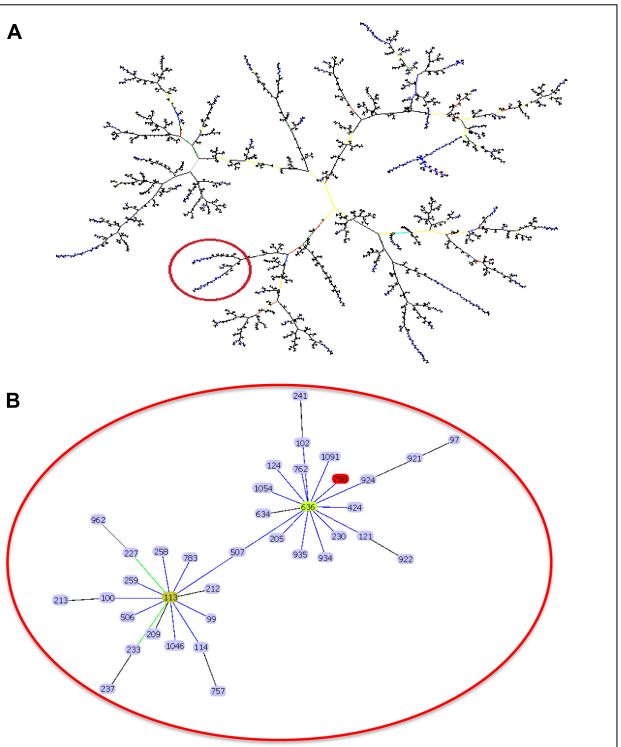

FIGURE 2 | eBURST analysis of *A. baumannii* strains. **(A)** Related and unrelated ST groups to the ST758 in *Acinetobacter baumannii* were compared with 1866 sequences (data generated by MLST) uploaded in the database. **(B)** Zoom showing to ST758 into the CC636.

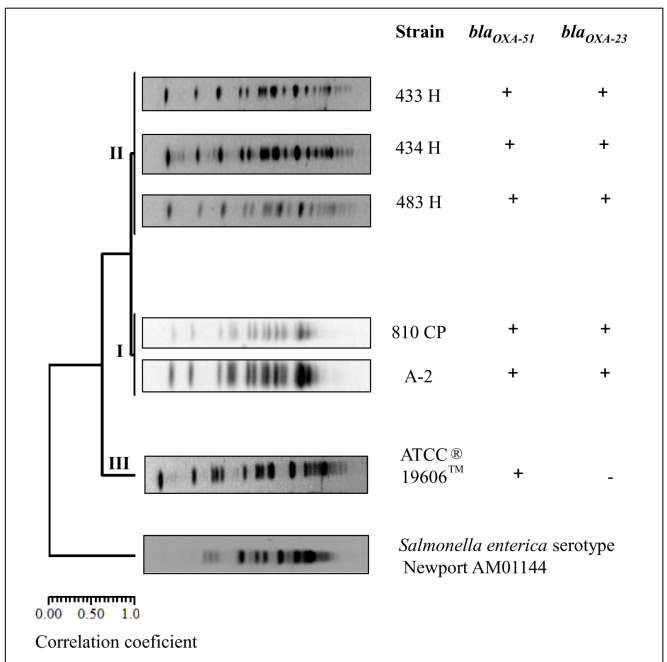

FIGURE 1 | Dendrogram profile displaying the genetic relatedness among the five *A. baumannii* strains based on the PFGE pattern, including the detection of bla_{oxa-51} and bla_{oxa-23} genes. *Salmonella enterica* serotype Newport AM01144 was used as external control.

A. baumannii Strains Could Adhere to, but Not Invade, A549 Cells

The adherence profile of the *A. baumannii* strain ATCC®19606™ toward A549 cells after a 4-h infection showed a mean value of 37.8×10^6 CFU/mL, significantly greater ($p = 0.001$) than the adherence profiles of the *A. baumannii* strains isolated in this study, which exhibited mean values between 23.3×10^6 and 30.6×10^6 CFU/mL (**Figure 3**).

In contrast, the invasion assay using *A. baumannii* ATCC®19606™ showed a 0.005% invasion frequency, which was significantly lower ($p = 0.028$) than the invasion frequency values of the *A. baumannii* strains isolated in this study (between 0.010 and 0.03%) (**Figure 3**). However, the *A. baumannii* strains and ATCC®19606™ showed lower invasion frequency values than other *A. baumannii* strains.

Sequencing of the Whole Genome of 810CP

To gain further insights into the genomic structure of *A. baumannii*, the complete genomic sequence of the 810CP strain was obtained using Illumina and PacBio platforms. Our analysis showed that the genome of 810CP consisted of a circular chromosome and two small plasmids (pAba810CPa and pAba810CPb). The general features of this genome are listed in **Table 3**.

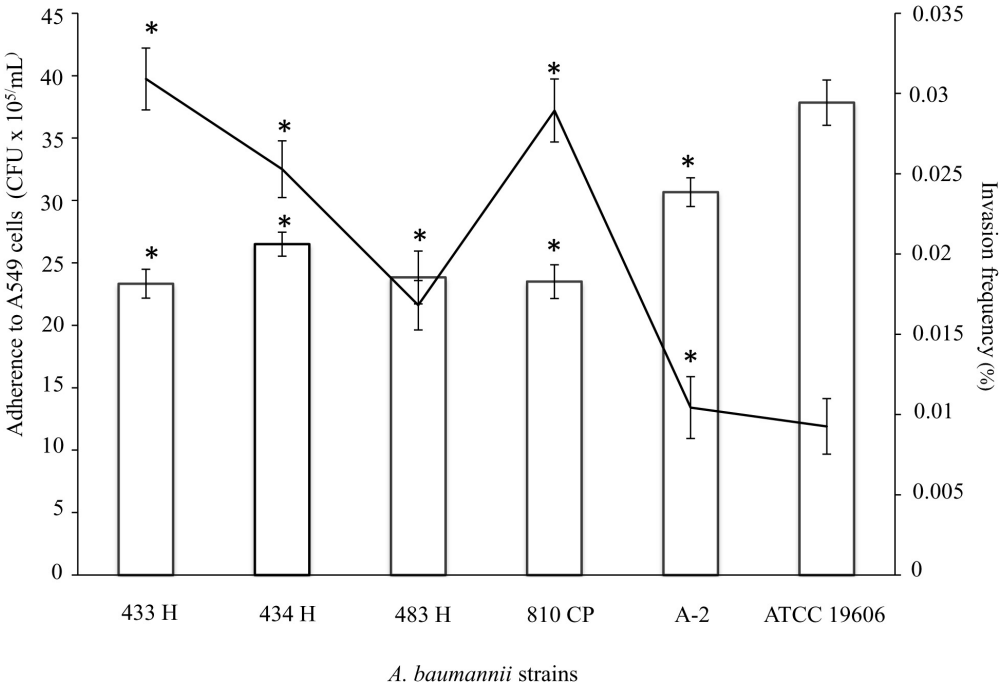

FIGURE 3 | Ability of *A. baumannii* strains to adhere and to invade A549 cells. Quantitative analysis of the adherence of *A. baumannii* strains to A549 cells showed significant increases ($p = 0.001$) after 4 h of infection. Analysis of the invasion frequency showed a significant increase ($p = 0.028$) comparing the 433H strain and other clinical strains, including ATCC® 19606™. The data (CFU/mL) for the invasion frequency are presented as the means and standard deviations from at least three independent experiments conducted in triplicate. *Represents a significant difference. The adherence data are presented as bars, and the invasion frequencies are black lines. *A. baumannii* ATCC® 19606™ was used as a positive control.

Genomic and Phage-Related Islands

The 810CP genome possesses a large number of IS elements, especially ISAba27, which is present at 16 chromosomal loci and occurs once in the largest plasmid pAba810CPb (**Table 3**, **Figure 4**, and **Supplementary Table 3**). This genome also contains 11 genomic islands, one of which encodes an essentially hypothetical protein. A 9996-bp resistance island carries genes that confer resistance to aminoglycoside and sulfonamide, as well as other genes encoding three transposases of different families associated with ISL3, IS6, and IS91 (**Table 3**). Seven regions containing phage-related genes are interspersed throughout the 810CP genome (**Figure 4**). The largest region is 49498 bp, which was present in only the 810CP strain among the *A. baumannii* genome sequences available in GenBank. Interestingly, these phage-related gene clusters are the most notable differences when comparing the 810CP chromosome sequence with other *A. baumannii* chromosomes sharing at least 90% coverage and 99% nucleotide identity (**Figure 4**).

Antibiotic and Heavy Metal-Resistance Genes

The 810CP genome has a wide range of genes involved in antibiotic and heavy metal resistance. The 810CP chromosome, like most *A. baumannii* strains, carries two genes (bla_{ACD-25} and bla_{OXA-65}) that are related to resistance to β-lactam antibiotics. The bla_{ACD-25} gene encodes an AmpC-type β-lactamase (cephalosporinase) that is adjacent to an ISAba1 element. The bla_{OXA-65} ($bla_{OXA-51-like}$) gene encodes a low-level carbapenamase. In the *A. baumannii* 810CP strain, a $bla_{OXA-239}$ gene was also identified, which is an allele belonging to the OXA-23 carbapenem-hydrolyzing class D family and is tightly linked to the ISAba1 element. This strain also contains the *strA* gene, which is involved in streptomycin resistance and associated with another ISAba1 element.

As previously mentioned, the 810CP genome possesses an antibiotic resistance island containing an *aac(6')-Ia* gene involved in resistance to aminoglycosides and a *sul2* gene linked to sulfonamide resistance. In addition, this strain carries a gene encoding an MdfA efflux pump and has two different chloramphenicol acetyltransferase genes, both of which are linked to a universal stress protein gene. The 810CP strain contains several genes involved in copper resistance that are interspersed throughout its genome. These genes encode proteins that include a TonB-dependent copper receptor (btuB_2), the copper-resistance protein CopB, and the copper-homeostasis protein NlpE.

Virulence-Associated Genes

Virulence genes in the 810CP strain were identified using the VFDB collection as queries in a BLASTn search, the results of which are listed in **Supplementary Table 4**. Briefly, in the 810CP genome, we identified the genes *lpxB*, *lpxD*, *lpxM*, and *lpsB*, which are involved in the synthesis of hepta-acylated lipooligosaccharides (LOS). Genes involved in capsule synthesis were identified within a large cluster in the 810CP chromosome

TABLE 3 | General features of the genome of *A. baumannii* 810CP.

Features	Chromosome	pAba810CPa	pAba810CPb
Length in bp	4,089,681	5,281	16,095
GC%	39.05	36.77	35.32
Number of protein-coding genes	3,812	8	18
Number of rRNA operons	6	0	0
Number of tRNA/ ncRNA/tmRNA genes	67/5/1	0	0
Number of insertion sequences (ISs)	ISAba1 (8)	ISAba27 (1)	0
	ISAba27 (16)		
	ISAba33 (8)		
	ISAba40 (1)		
	ISAba43 (1)		

(**Supplementary Table 4**). Based on the sequencing analysis, we also identified the *pgaABC* locus, encoding proteins involved in the production of poly-β-1-6-*N*-acetylglucosamine, as well as the *ompA* gene, encoding a major component of the outer membrane protein (OmpA), a trimeric porin involved in solute transport and biofilm formation. Other genes with a role in biofilm formation and involved in the synthesis of the Csu pili and the two-component system BfmS were also identified. The 810CP genome also possesses a gene encoding Bap (biofilm-associated protein, locus-tag Aba810CP_04235), a protein with multiple immunoglobulin-like domains localized on the cell surface. Genes encoding the AdeFGH resistance–nodulation–cell division (RND)-type efflux system were identified using the VFDB. In the chromosome of *A. baumannii* 810CP, two genes (locus-tag Aba810CP_02675 and Aba810CP_09700) were identified encoding the phospholipase D family and two types of phospholipase C, one gene (locus_tag Aba810CP_19600) with a match in the VFDB, and a third gene annotated only as "phospholipase."

In addition, we identified a gene encoding PbpG (locus_tag Aba810CP_18695) and genes involved in the synthesis of two iron-uptake systems mediated by the siderophore acinetobactin.

Plasmids

Acinetobacter baumannii 810CP possesses two plasmids: pAba810CPa (5281 bp) and pAba810CPb (16095 bp) (**Figure 4**). The plasmid pAba810CPa harbors only six predicted ORFs, one

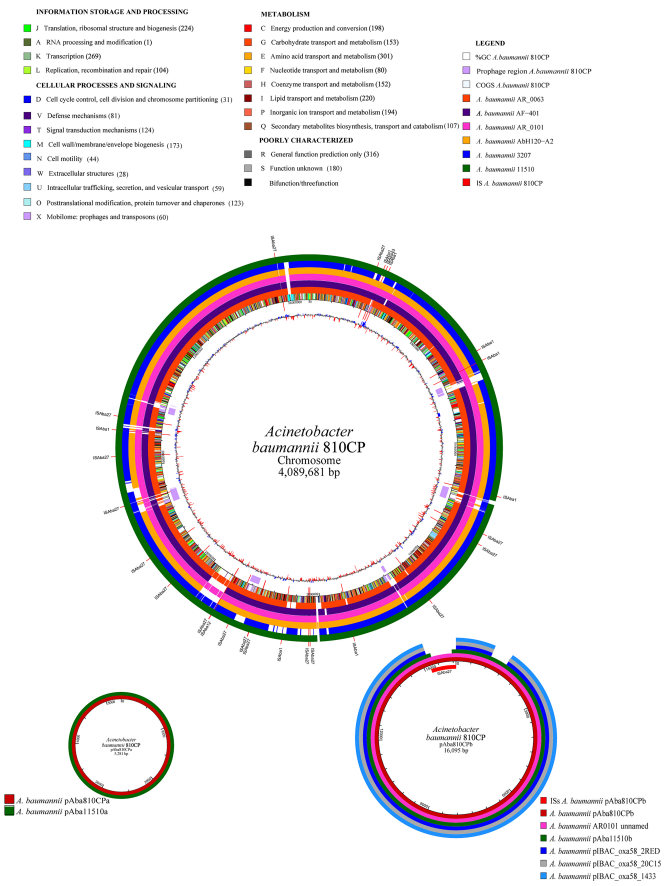

FIGURE 4 | Comparative genome analysis of the *A. baumannii* 810CP strain. The functional classes are represented in different colors. The number of proteins identified for each functional class is shown in parentheses. The *A. baumannii* genome and plasmids that were compared with the *A. baumannii* 810CP strain are shown in different colors.

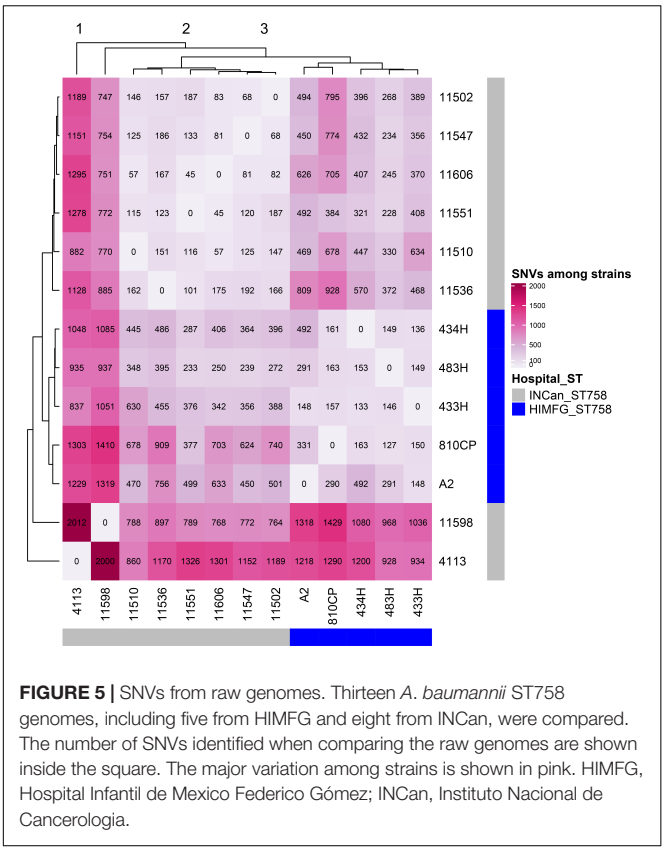

FIGURE 5 | SNVs from raw genomes. Thirteen *A. baumannii* ST758 genomes, including five from HIMFG and eight from INCan, were compared. The number of SNVs identified when comparing the raw genomes are shown inside the square. The major variation among strains is shown in pink. HIMFG, Hospital Infantil de Mexico Federico Gómez; INCan, Instituto Nacional de Cancerologia.

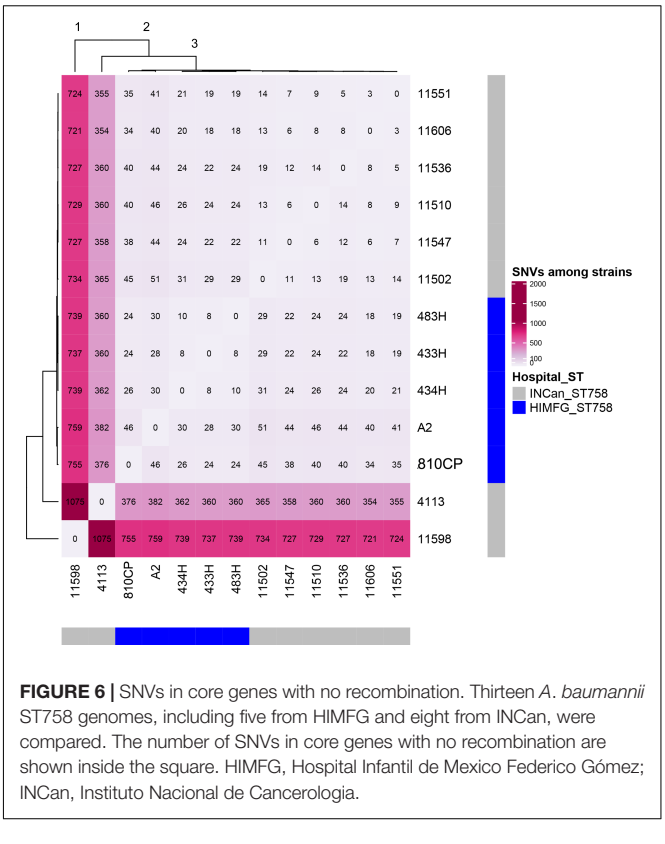

FIGURE 6 | SNVs in core genes with no recombination. Thirteen *A. baumannii* ST758 genomes, including five from HIMFG and eight from INCan, were compared. The number of SNVs in core genes with no recombination are shown inside the square. HIMFG, Hospital Infantil de Mexico Federico Gómez; INCan, Instituto Nacional de Cancerologia.

of which encodes RepB, a protein containing a Rep_3 motif involved in plasmid replication (replicase). This plasmid also possesses genes involved in plasmid mobilization and those related to toxin–antitoxin (TA) systems, although antibiotic-resistance genes were not identified. The pAba810CPb plasmid has 18 predicted ORFs, 8 of which are annotated as hypothetical proteins. Similar to pAba810CPa, pAba810CPb has a TA system (**Figure 4**). Considering that this plasmid harbors a relaxase-encoding gene (*traA*), it is likely that pAba810CPb is a mobilizable plasmid. pAba810CPb does not encode genes involved in antibiotic resistance, and it contains an integrated ISAba27 element between a TonB-dependent receptor gene and a septicolysin gene. This plasmid also carries genes with a general classification: a CopG family transcriptional regulator, a DNA binding protein, and an acetyltransferase protein. A replicase gene was not identified, indicating that this plasmid has a novel replication system.

Comparative Genomics of *A. baumannii* ST758 Strains

The remaining four *A. baumannii* strains (433H, 434H, 483H, and A-2) isolated from same patient were sequenced using only an Illumina platform, and the resulting sequences of these isolates were compared. Analysis of the sequences showed that the strains are closely related but not identical. The five isolates have the same ST (ST758) and possess the same antibiotic resistant genes in the same relative positions. The number of SNVs observed in the complete genomes varied between 127 and 492 (**Figure 5**), but when only the SNVs from the core genome without recombination signals were calculated, significantly fewer SNVs were identified, ranging from 8 and 30 (**Figure 6**). Strains 433H, 434H, and 483H varied by 8–10 SNVs, indicating that they are more closely related with each other than with strains A2 and 810CP. However, more dramatic differences were observed in gene content. The five strains have a pangenome of 3811 genes, but their core genome is made up of only 3613 genes. In other words, the non-core genome consists of 198 genes (**Figure 7**). This observation indicates that rapid gene turnover is crucial for generating genetic diversity in *A. baumannii*, as proposed by Graña-Miraglia et al. (2017). When the genome sequences of other ST758 strains isolated from a different Mexican tertiary care hospital (INCan) were included in the analysis, the variation in SNVs in the complete genomes revealed that the strains 4113 and 11598 showed the greatest variation in the number of SNVs relative to the rest of the assayed strains. It has been proposed that these two strains are hypermutators, and they possess mutations in genes involved in DNA repair (Graña-Miraglia et al., 2017). Interestingly, all the strains isolated from the same patient were grouped together, while the other ST758 strains clustered into the other group (**Figure 5**, **6**). When the gene content of all ST758 strains was analyzed, the number of genes included in the pangenome increased to 4010 genes, while the number of genes in the core genome decreased to 3422 genes (**Figure 7**). Subsequently, an analysis using BacWGSTdb showed that all of our strains had the same resistance genes and were

ST758. However, some variations were observed with respect to virulence genes. Around 80 virulence genes were present in most strains (**Supplementary Table 4**). However, the strains 810CP and 483H contain only 44 and 77 virulence genes, respectively. Interestingly, the virulence gene data set for the 810CP strain was identical to that of the 11510 strain (an ST758 isolate from INCan).

DISCUSSION

Acute leukemia is the most common cancer in children younger than 15 years old and has a wide-ranging incidence worldwide, with this disease being particularly prevalent in Hispanic residents in the United States and in Mexican children (Pérez-Saldivar et al., 2011; Metayer et al., 2013). *A. baumannii* is a major cause of morbidity and mortality due to the occurrence of new MDR clinical strains. The risk factors for *A. baumannii* infections, bacteremia, and colonization include severe underlying illness (particularly in critically ill patients and those with hematologic malignancy), prolonged antibiotic therapy with broad spectrum antibiotics, urinary tract infections and catheterization, respiratory tract colonization, infection and endotracheal intubation, and intestinal colonization (Cisneros et al., 1996; Maragakis and Perl, 2008). The overall mortality associated with *A. baumannii* bacteremia ranges between 25 and 54% and depends on the physical condition of the patient. Even in critically ill patients, some studies have shown that the contribution of MDR to *A. baumannii* bacteremia varies between 7.8 and 19%. Septic shock has been reported in up to 42% of patients with bacteremia and crude MDR in patients with bacteremia may reach 70%. However, complicated clinical courses and life-threatening complications are more likely to occur in immunocompromised individuals (Cisneros et al., 1996; Maragakis and Perl, 2008).

Infections associated with carbapenem-resistant *A. baumannii* have been associated with high mortality rates, prolonged hospital stays, and increased health costs (Song et al., 2011; Lee et al., 2014). To nosocomial bacteria such as *A. baumannii* strains, an MDR profile confers a high probability of surviving in a hospital environment and is associated with hospital outbreaks (Cheng et al., 2015; Villalon et al., 2015). Our results characterized the *A. baumannii* strain 810CP as MDR, with the same MIC observed for imipenem and meropenem. Resistance to this antibiotic has increased in recent years worldwide, and in a tertiary care hospital in México, 89.2% of *A. baumannii* strains was observed to be resistant to imipenem (Rosales-Reyes et al., 2017), similar to reports in other countries (Liu et al., 2018).

In the 810CP genome, we identified the AdeFGH RND-efflux system, the overexpression of which confers MDR and the ability to pump antibiotics out of the cell. Furthermore, this system increases biofilm formation together with the overexpression of *adeG*, a component of this system (Coyne et al., 2010). Interestingly, the ISAba1 element identified in the 810CP genome has been previously observed to be associated with the *strA* gene, the encoded product of which confers streptomycin resistance, and it is also involved in promoting ISAba1 overexpression and bacterial susceptibility to these antibiotic classes (Corvec et al., 2003, 2007; Mugnier et al., 2009). The gene encoding the MdfA efflux pump was detected in the 810CP genome, the overexpression of which has been associated with resistance to several antibiotics, such as chloramphenicol and ciprofloxacin (Vila et al., 2007). Other important findings by our group include the identification of a 9996-bp resistance island that has recently been described in other *A. baumannii* strains [e.g., AR0101 (GenBank CP027611.1), 11510 (Graña-Miraglia et al., 2017),

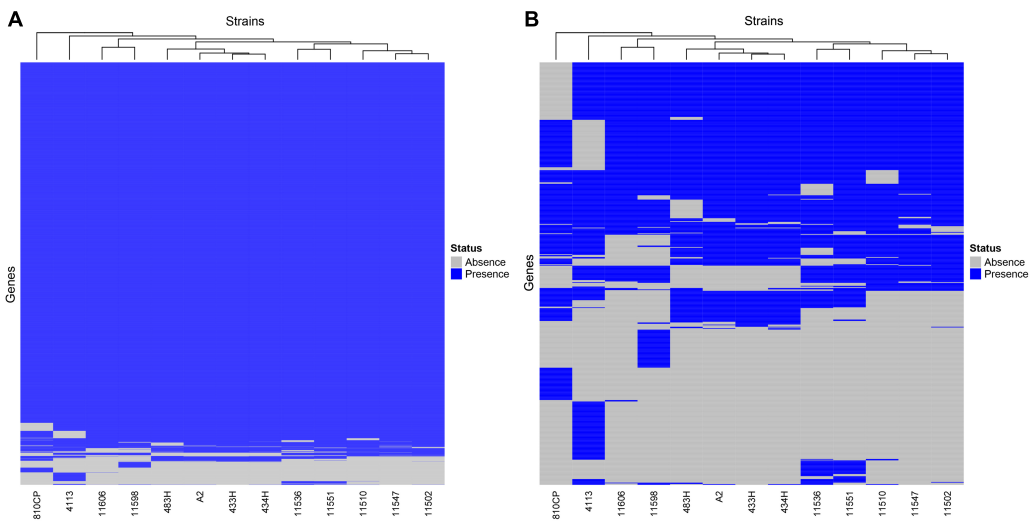

FIGURE 7 | Core and non-core gene heatmaps of *A. baumannii* strains. The genomes of 13 ST758 *A. baumannii* strains, including 810CP, were compared. **(A)** Core and non-core genes identified in all strains. **(B)** Non-core genes identified in all strains. The genes identified among all strains are shown as follows: the presence or absence of these genes in the genomes are shown in blue and gray, respectively.

AF401 (GenBank NZ_CP018254.1), AbH120-A2 (Merino et al., 2014), and AB030 (Loewen et al., 2014)].

The bla_{OXA-23} gene has been considered an important resistance biomarker and is located in either the plasmid or chromosome and is highly prevalent (Mugnier et al., 2010; Opazo et al., 2012; Evans and Amyes, 2014; Luo T.L. et al., 2015). MLST analyses have identified several clonal complexes, groupings of unique genotypes that share at least five loci, and these genotypes have diversified and increased their frequency in the population. For instance, the CC92B clonal complex, which is rarely observed in Latin American isolates but is prevalent in Asia and the United States, is found worldwide and includes carbapenem-resistant clones that harbor the bla_{OXA-23} gene encoding OXA-23, representing a risk of propagation of resistant clones (Ji et al., 2013; Wang et al., 2013; Gonzalez-Villoria et al., 2016). Some studies have described genes encoding OXAs that are located in plasmids. However, the bla_{OXA-23} gene described by our group was not identified in any of the plasmids studied; therefore, this OXA is encoded in the chromosome (Mugnier et al., 2010). Plasmid pAba810CPa, harboring the gene encoding RepB, contains identical characteristics to plasmid pAba11510a, which was described in another Mexican *A. baumannii* strain belonging to ST758 (Graña-Miraglia et al., 2017). The pAba810CPb plasmid identified in the 810CP strain has a TA system that can enhance the survival of bacterial cells during infection (Díaz-Orejas et al., 2017). Interestingly, genes encoding a TonB-dependent receptor and septicolysin are present in pAba810CPb adjacent to each other. However, many plasmids are not closely related to pAba810CPb, such as pAC12, pAC30a, pAC29a, pAB0057, p2ABYE, pMV01, and pAbaATCC233 (Lean et al., 2016).

In this study, similar PFGE and MLST profiles were obtained for the analyzed *A. baumannii* strains, and the genetic difference of a 388-kb fragment was identified only by PFGE analysis in the strains isolated from the bloodstream compared with the stool and autopsy strains (Tenover et al., 1995). Additionally, five ST758 strains identified by MLST sequencing of bla_{OXA-23} (data not shown) and WGS were primarily associated with the OXA-239 allele belonging to the OXA-23 group (Gonzalez-Villoria et al., 2016).

To establish if the five strains were closely related, a comparative genomic analysis was performed. The complete genome sequence of the 810CP strain was obtained using the Illumina and PacBio platforms, while the remaining four *A. baumannii* strains were sequenced only with the Illumina platform. An analysis using BacWGSTdb showed that all of our strains were ST758, as shown by the MLST analysis. Interestingly, ST758 belonging to CC636 corresponds to the Ibero American complex, which has been reported to be the most widespread in Europe, Asia, South Africa, and the United States (Tamayo-Legorreta et al., 2014; Lowings et al., 2015; Vanegas et al., 2015; Gonzalez-Villoria et al., 2016). In Colombia, CC636 has been considered a high-risk clone due to its frequent association with MDR *A. baumannii* strains, including carbapenem resistance (Correa et al., 2018).

Comparative genomic analysis was performed among 13 strains isolated from two Mexican hospitals (HIMFG and INCan), all belonging to ST758. The core genome of these strains consists in 34422 genes (86% of the pangenome). The SNVs analysis showed differences among the raw genomes and the genes that are not subject to recombination. Because recombination contributes changes in the genome, is better to use genes that are not subject to recombination to establish relationship among strains. In other bacteria, pairs of sequences varying by 2 SNVs were considered sufficiently closely related to be compatible with a recent direct transmission/acquisition from a common source. Pairs of sequences varying by 0–10 SNVs were considered related through a shared common ancestor sometime during or shortly before the study (∼5 years evolution). Pairs of sequences varying by >10 SNVs were considered genetically distinct (Eyre et al., 2013). Interestingly, the amount of gene content variation in the *A. baumannii* strains was higher than the number of SNVs. These differences observed in our strains can be interpreted in two ways: first, all strains were genetically different, even if they had been isolated from the same patient, and in some cases from the same sample site, suggesting that the patient was infected by a number of distinct, but closely related strains. The other interpretation is that all genetic differences were acquired during the patient illness, indicating, as suggested by Graña-Miraglia et al. (2017), that the A. *baumannii* genome is highly dynamic and that gene turnover may have a crucial role shaping the genome of this microorganism. Additional studies must be performed to clarify this point.

The interface between *A. baumannii* and its environment is the cell envelope and includes the capsule. Genes involved in capsular biosynthesis were identified in our strains. *A. baumannii* cells possess a thick capsular polysaccharide to protect them from different external stresses, including desiccation and host defenses. *A. baumannii* strains lacking the capsule are non-virulent and are affected in biofilm formation (Lees-Miller et al., 2013). Genes involved in the synthesis of hepta-acylated LOS were identified in the genome. *A. baumannii* with LOS mutations are viable, but *in vitro*, they develop growth defects, resulting in severely diminished virulence (Beceiro et al., 2014; Powers and Trent, 2018). The five *A. baumannii* strains and the ATCC®19606™ strain with adhesion values to A549 cells ranged from 6.4×10^4 to 4.5×10^5 CFU/mL, similar to values reported in other studies (Eijkelkamp et al., 2011; Giannouli et al., 2013; Na et al., 2016; Ambrosi et al., 2017; Pérez et al., 2017). Sequencing of the whole genomes revealed genes involved in the biogenesis of the type IV pilus, which in Gram-negative bacteria, promotes adherence to human epithelial cells and the formation of microcolonies. However, the role of type IV pili in the *A. baumannii* remains to be investigated (Knutton et al., 1999; Barken et al., 2008; Saldaña-Ahuactzi et al., 2016).

Interestingly, the genes encoding BfmR/BfmS are also present in the genomes. The pili of *A. baumannii* are encoded by the csuA/BABCDE chaperone-usher assembly system, which is controlled by a two-component regulatory system (BfmS and BfmR). This pilus has been associated with twitching-motility and biofilm formation by QS signaling molecules that enhance the expression of the chaperone-usher secretion system (Luo L.M. et al., 2015). The five *A. baumannii* strains obtained from the child with leukemia M2 formed biofilms on polystyrene

surfaces when cultured in tryptone soy broth for 24 h at 37°C (data not shown). Biofilm formation and the acquisition of antibiotic-resistance genes in *A. baumannii* are properties that allow this pathogen to survive within a nosocomial environment (Roca et al., 2012). Mutations in the *bfmR*, *bfmS*, and *bap* genes result in decreased or disrupted biofilm formation, while the absence of the *bap* gene decreases adherence to human bronchial cells (Loehfelm et al., 2008; Tomaras et al., 2008; Brossard and Campagnari, 2012). The *pgaABCD* locus in *A. baumannii* encodes poly-β-1-6-*N*-acetylglucosamine, a molecule that plays a role in biofilm formation (Choi et al., 2009). In other pathogens, this molecule plays a major role in cell-to-surface and cell-to-cell adherence and protects cells against host defense mechanisms (Vuong et al., 2004; Sivaranjani et al., 2018). A mutation in the *abaI* gene that encodes the acyl-homoserine lactone autoinducer significantly affects biofilm formation and is favored following the addition of purified acyl-homoserine lactone or overexpression of the *abaI* gene (Bhargava et al., 2010).

Acinetobacter baumannii invades epithelial cells via a zipper-like mechanism, which is associated with microfilament and microtubule-dependent uptake mechanisms (Choi et al., 2008). Our data showed that epithelial cells derived from the respiratory tract were more susceptible to *A. baumannii* invasion than non-respiratory tract-derived epithelial cells. However, the strains analyzed in this study showed a low frequency of invasion compared with other invasive pathogens, such as *E. coli*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, and *Cronobacter* species (Fleiszig et al., 1995; Huang et al., 1999; Cruz et al., 2011; Uliczka et al., 2011). Recently, OmpA and phosphorylcholine-porin D have been shown to be associated with cell adherence, invasion, and survival within pneumocytes (Mortensen and Skaar, 2012; Ambrosi et al., 2017).

The chromosomes of the assayed *A. baumannii* strains had five genes encoding phospholipases with the ability to hydrolyze phospholipids and contribute to pathogenesis, including the enzymatic activity that occurs during lysis of the host cell membrane (Antunes et al., 2011). These enzymes play a role in the infection and invasion of eukaryotic host cells (Stahl et al., 2015). Phospholipase C has been shown to play a role in virulence in several pathogens, including *A. baumannii*. Recently, phospholipase C in conjunction with elastase has been shown to enhance virulence in an insect model (Kareem et al., 2017).

In the *A. baumannii* genomes, two iron-uptake systems and a gene cluster involved in heme utilization were identified that are important for virulence, as demonstrated in other pathogens (Hom et al., 2013; Ou et al., 2015). Iron is a scarce element in the mammalian host, although it is essential for pathogen survival and infectivity. The systems involved in iron acquisition are very important for virulence. These systems are present in *A. baumannii* genomes and are essential for growth under iron-limiting laboratory conditions (Gaddy et al., 2012). The bioinformatics analysis of the genomes revealed that the TA systems that form a complex in which the antitoxin inhibits toxin activity are encoded by plasmids or chromosomes (Leplae et al., 2011). TA systems have been suggested to mediate bacterial persistence by generating slowly growing cells that are tolerant to antibiotics and environmental changes. Moreover, these systems promote biofilm formation through programmed cell death (Yamaguchi and Inouye, 2009; Fair and Tor, 2014). At least five different TA systems have been identified based on the genomic sequencing of *A. baumannii* strains, including Sp1TA (DUF497/COG3514) (Fernández-García et al., 2016). Among a collection of *A. baumannii* clinical strains from Lithuanian hospitals (88.6% prevalence), HigB/HigAAb and SplTA TA systems were identified as the most abundant. These noncanonical TA systems are most prevalent in clinical *A. baumannii* strains belonging to the ECI and ECII lineages, which are widespread worldwide (Jurenaite et al., 2013). Among five strains, 810CP strain was the most different in relation to virulence genes, this could be associated to this origin, and that it was the first isolated from the child.

In summary, the patient described in this study was severely immunocompromised due to chemotherapy treatment and to the deterioration of health, increasing the risk of developing an infection by *A. baumannii*. According to molecular typing results, the strains showed identically PFGE and ST profiles. However, the results of genome sequencing determined that they were different strains with a closely related origin. The *A. baumannii* strains described in this study showed important characteristics meriting further investigation, such as with respect to pathway genomics, resistance, and surveillance of molecular epidemiology. The identified ST758 in the *A. baumannii* strains and the associated $bla_{\mathrm{OXA-23}}$ gene are considered genetic biomarkers that contribute to the persistence of these bacteria in the hospital environment. However, further assays are needed to determine whether these strains were endemic to our hospital or had evolved over time. Therefore, genetic studies are required to demonstrate the contribution of putative genes involved in the virulence of *A. baumannii*.

ETHICS STATEMENT

Research Committee (Dr. Juan Garduño Espinosa), Ethics Committee (Dr. Luis Jasso Gutiérrez), and Biosecurity Committee (Dr. Marcela Salazar García) of Hospital Infantil de México Federico Gómez (HIMFG) granted the approval for the development of the protocol HIM/2017/003 SSA.1299. Written informed consent was not required for this study according to the institutional ethical, biosecurity, and investigation Committee due to Central Laboratory from HIMFG provided the *A. baumannii* clinical strains isolates from the child included in this study.

AUTHOR CONTRIBUTIONS

AC-C had the initial idea, which was developed into a project together with JX-C and JM-R. JM-R performed the experiments. AC-C, JX-C, MAC, SAO, VML-P, and JA-G analyzed the data. SC-J, MAC, and PB assembled, annotated, and performed bioinformatics analysis of genomes. AL-G reviewed and described the clinical case. MBdV supported the MLST sequencing. AC-C, JX-C, MAC, SAO, JA-G, MBdV, and RH-C contributed reagents and materials.

IP-O supplied the *A. baumannii* strains. AC-C, JX-C, and MAC wrote the manuscript, and read and approved the final version. All authors discussed and corrected the manuscript and approved it for publication.

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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.2019.00132/full#supplementary-material

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Apéndice 7 - Structure and Evolution of *Acinetobacter baumannii* Plasmids.

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Structure and Evolution of *Acinetobacter baumannii* Plasmids

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Acinetobacter baumannii is an emergent bacterial pathogen that provokes many types of infections in hospitals around the world. The genome of this organism consists of a chromosome and plasmids. These plasmids vary over a wide size range and many of them have been linked to the acquisition of antibiotic-resistance genes. Our bioinformatic analyses indicate that *A. baumannii* plasmids belong to a small number of plasmid lineages. The general structure of these lineages seems to be very stable and consists not only of genes involved in plasmid maintenance functions but of gene sets encoding poorly characterized proteins, not obviously linked to survival in the hospital setting, and opening the possibility that they improve the parasitic properties of plasmids. An analysis of genes involved in replication, suggests that members of the same plasmid lineage are part of the same plasmid incompatibility group. The same analysis showed the necessity of classifying the Rep proteins in ten new groups, under the scheme proposed by Bertini et al. (2010). Also, we show that some plasmid lineages have the potential capacity to replicate in many bacterial genera including those embracing human pathogen species, while others seem to replicate only within the limits of the *Acinetobacter* genus. Moreover, some plasmid lineages are widely distributed along the *A. baumannii* phylogenetic tree. Despite this, a number of them lack genes involved in conjugation or mobilization functions. Interestingly, only 34.6% of the plasmids analyzed here possess antibiotic resistance genes and most of them belong to fourteen plasmid lineages of the twenty one described here. Gene flux between plasmid lineages appears primarily limited to transposable elements, which sometimes carry antibiotic resistance genes. In most plasmid lineages transposable elements and antibiotic resistance genes are secondary acquisitions. Finally, broad host-range plasmids appear to have played a crucial role.

Keywords: *A. baumannii*, plasmids, Rep proteins, antibiotic resistance genes, plasmid maintenance functions, IS

INTRODUCTION

Acinetobacter baumannii is a global emergent nosocomial pathogen that causes a wide variety of infections, especially in severely ill patients, in intensive care units. This pathogen is a major cause of morbidity and mortality in hospitals worldwide, and the recent success of this species as a pathogen seems to be linked to the ability of this organism to acquire antibiotic resistance genes, form biofilms and resist desiccation; these characteristics facilitate the persistence of this bacterium in the hospital setting and promote the emergence of outbreaks (Antunes et al., 2014). A large fraction of the nosocomial outbreaks in Europe, Asia, and North America are produced by a limited number of strains belonging to three different international clones (IC-I, IC-II, and IC-III) (Zarrilli et al., 2013). Most of these international clones are resistant to antibiotics belonging to three or more different families, a characteristic that defines these clones as being multidrug resistant (MDR) (Diancourt et al., 2010; Roca et al., 2012).

Plasmids are extrachromosomal DNA molecules, usually circular, that replicate independently of the chromosome and have the potential to be transferred frequently, but not exclusively by conjugation, not only to members of the same species but also to distantly related bacteria (Partridge et al., 2018). Plasmids play a leading role in the spread of antibiotic resistance genes among bacterial pathogens that cause community- or hospital-acquired infections, including *A. baumannii* (Carattoli, 2013; San Millan, 2018). A wide variety of *A. baumannii* plasmids carrying antibiotic resistance genes with different sizes and characteristics have been described in recent literature. There has been particular interest in plasmids carrying genes encoding serine carbapenemases (OXA-type beta-lactamases), which facilitate the most predominant mechanism for carbapenem resistance in this species (Higgins et al., 2010; Mosqueda et al., 2014; Hujer et al., 2017; Cameranesi et al., 2018; Wibberg et al., 2018).

Despite the apparent importance of plasmids in the spread of virulence and antibiotic resistance genes among *A. baumannii* isolates, only a few papers that have analyzed the structures, relationships and evolution of *A. baumannii* plasmids as a whole have been published (Fondi et al., 2010; Lean and Yeo, 2017; Salto et al., 2018). In this work, taking advantage of the increasing interest in *A. baumannii* and the large number of complete genome sequences for this organism that have been deposited in GenBank in the last decade, we performed a comparative plasmid sequence analysis to gain insights into the structures, relatedness, and evolution of these plasmids. We were able to determine that the *A. baumannii* plasmids belong to a small number of plasmid lineages, some of them widely distributed among the different *A. baumannii* clades, while others seem to be restricted to a small number of clades. Surprisingly, some widespread plasmids do not have genes linked to conjugation or plasmid mobilization, suggesting that other mechanisms or horizontal transfer play an important role in the dissemination of *A. baumannii* plasmids. Genes encoding initiator replication proteins and the corresponding surrounding DNA sequences within each plasmid lineage are similar enough to suggest that each lineage represents plasmids of the same incompatibility group. This suggestion is also supported by the observation that plasmids of the same strain have different replication proteins. Each plasmid lineage possesses a common gene set that contains not only genes involved in plasmid maintenance but also a set of genes encoding hypothetical or poorly characterized proteins. Despite the antibiotic or metal resistance genes that some plasmids possess, the remaining genes that are not involved in plasmid maintenance are not obviously linked with properties that allow survival in the hospital setting, suggesting that these genes could be associated with plasmid survival functions. Additionally, we determined that gene transfer from one plasmid lineage to another is highly limited and restricted to a few gene classes.

RESULTS AND DISCUSSION

The Plasmid Collection

Next-generation sequencing platforms have been a crucial means to obtain the complete sequences of all types of bacterial genomes, including those of many important human pathogens. We took advantage of the large amount of information generated in this manner to analyze the structure and evolution of the *A. baumannii* plasmids. For this purpose, we used the 155 complete plasmid sequences deposited in NCBI as of August 14, 2017. However, considering that most of these plasmid sequences were obtained from isolates of international clones and/or from a restricted set of countries, we incorporated the sequences of 18 plasmids obtained from the genome sequences of 10 nosocomial strains that represent some of the most prevalent STs circulating in Mexico to increase the plasmid diversity included in our investigation (see Materials and Methods). In total, our study collection comprised 173 plasmids of a wide variety of sizes, ranging from 1,109 to 216,780 bp. Moreover, our plasmid set originated from 103 different isolates, each carrying up to six plasmids. These isolates belonged to at least 47 different STs and originated from 17 countries (see **Supplementary Table S1**).

A. baumannii Plasmids Belong to a Very Restricted Number of Plasmid Lineages

Plasmids have been visualized as molecules that possess genes involved in self-maintenance (plasmid backbone) and genes that could be important for the ability of bacteria to exploit new ecological niches or acquire new capabilities (Frost et al., 2005). These genes are commonly described as plasmid *cargo*. Antibiotic resistance genes are a perfect example of such genes, particularly for organisms in hospital settings (Tschäpe, 1994; Carattoli, 2013; San Millan, 2018).

To understand how plasmids are organized and to define which are the relationship between them, several plasmid classification systems have been proposed. Some of these systems relay in the phenotypic features that plasmids confer, assuming that plasmids sharing such characteristics are phylogenetically related. Plasmid incompatibility or the inability of two plasmids to reside in the same cell has been another way to classify plasmids. Plasmids belonging to the same incompatibility group have identical or very similar replication and/or segregation gene

modules (Novick, 1987; Austin and Nordström, 1990). With this idea in mind, some authors have developed typing systems based on the nucleotide sequence identity of the genes encoding replication initiation proteins. Other authors designed methods to classify conjugative plasmids based on the sequence of the relaxase, a gene crucial for conjugation. The problem with these classification systems is that they are based on a limited number of genes or traits. However, considering the diversity of genes carried on plasmids and the different mechanisms that plasmid use for their maintenance makes a futile dream to design a universal plasmid taxonomy system. Nevertheless, we can design a classification system that takes into account, in an unbiased way, the whole gene content of plasmids, to determine which are the relationships between them and to have a picture of how these plasmids evolve. This was the approach that we follow in this work.

Plasmid evolution can be thought to occur via two basic pathways: first, plasmids are entities that are prone to rapid loss and gain of genes such that, in a short period of time, descendants of one plasmid are only recognizable because they share the same set of genes involved in the basic maintenance functions of the plasmid (Hülter et al., 2017; Brandt et al., 2019). Second, the ability of plasmids to gain or lose genetic information can be assumed to be more or less limited, and the plasmids persist for long durations within bacterial populations as plasmid lineages, where plasmid lineages are groups of plasmids that are closely related by gene content, including, but not restricted to, genes responsible for plasmid maintenance (Yau et al., 2010).

Our first interest was to evaluate, precisely, the type of evolution undergone by *A. baumannii* plasmids. For this purpose, our strategy was to compare the degree and extent of DNA sequence identity between the plasmids in our collection. We used nucleotide MEGABLAST (BLASTn) searches instead of Protein BLAST (BLASTp), as described by other authors, for two reasons: first, BLASTn comparisons are less sensitive to sequencing errors introduced during the assembly process (false frame shifts or incorrect stop codons) than BLASTp, and second, a BLASTp approach does not take into consideration intergenic regions and regions essential for plasmid function, such as the origin of replication. We made pairwise MEGABLAST (BLASTn) comparisons of each plasmid of our collection against the others. To filter BLAST results, we constructed networks with the following rule: two plasmids are linked if at least 85% of the regions of the largest plasmid (for each comparison) are covered by the smaller plasmid, and those regions exhibit at least 90% of DNA sequence identity. To belong to a specific network, one plasmid must fulfill the above-mentioned cutoff values of identity and coverage not with all, but with at least one member of the group. Being a member of a specific network does no mean that all plasmids of this network have at least 85% coverage with the rest of the members. The minimal requirement is to accomplish the cutoff values with at least one member of the network, for example, the shortest with the next in size.

After these analyses, we determined that 124 *A. baumannii* plasmids were organized into 23 groups, and 39 plasmids remained without an assigned group. The plasmid composition of each group is listed in **Supplementary Table S1**. As shown in **Supplementary Figure S1**, the plasmid networks constructed as mentioned above are densely interconnected, and all members of a determined group have the same or a very closely related gene encoding a DNA replication initiator (Rep) protein. Notably, plasmids within a group share, in general, several genes that are involved in plasmid maintenance.

To evaluate the coherence of these groups, we repeated the analysis, raising plasmid coverage to 90% again, with 90% DNA sequence identity. In general, the groups remained almost the same (some groups lost a few members). On the one hand, lowering coverage to 50% and retaining 90% of DNA sequence identity, allows the incorporation of some orphans into different groups and led to the fusion of six lineages: Group_17 with Group_22, Group_7 with Group_8 and Group_3 with Group_14. Members of lineages Group_17 share sequence identity of approximately 70% with components of Group_22, including the replication module and nearby sequences. This grouping suggest that Group_17 and Group_22 have a common evolutionary origin. Likewise, members of Group_3_and Group_14 have similar but not identical Rep proteins, indicating also that they have a hypothetical common ancestor. In contrast, members of Group_8 do not have the same replication module as those belonging to Group_7 and for this reason we do not contemplate them having a common ancestry.

Groups formed using 85% coverage and 90% of sequence identity as cutoff values represent a useful method for identification of *A. baumannii* plasmid lineages. Lowering the coverage cutoff value to 50% may be useful to recognize ancestral relationships, as long as the shared sequences include the replication/maintenance module. Therefore, hereinafter, we will consider each one of the groups identified with this methodology as a plasmid lineage.

However, to indicate that Group_3_and Group_14 had a common origin but now each one of the groups has a different evolutionary path, these were named as plasmid lineages LN_3A and LN_3B, respectively. With these considerations, members of our plasmid collection belong to 21 plasmid lineages and 39 plasmids remain as orphans (not assigned to a plasmid lineage). Interestingly, 88 plasmids, or 50.8% of our collection, were clustered in only four plasmid lineages: LN-1, LN_2; LN_3 and LN_4. The other 17 groups are very small, as most of them contained only two members (**Supplementary Figure S1**).

With only one exception, we elected the largest and most interconnected member of the group as the representative plasmid of each lineage. The exception is lineage 2 (LN_2), in which the largest and most interconnected member has a very large duplicated region. The duplicated regions include the replication genes indicating that this sequence has assembly problems, considering that plasmids with duplicated replication regions are highly unstable and they are rapidly eliminated of the population (Summers et al., 1993). Therefore, the second largest most interconnected plasmid (pPKAB07) was selected as the representative of this particular lineage. In conjunction, these analyses indicate that *A. baumannii* plasmids evolve as lineages

and that most of the *A. baumannii* plasmids in circulation worldwide belong to a few lineages.

The general structure of the members of each one of the plasmid lineages is very stable, considering that some of the strains were isolated many years ago. For example, strain A1 was isolated in 1982, and one of the plasmids of this strain, pA1-1, belongs to lineage LN_2. This plasmid has a very similar gene content and organization as other plasmids isolated in 2015 that belong to the same lineage (plasmid unnamed2, GenBank accession number CP014293). Similarly, plasmid pALAC4-2 of LN_4 belongs to a strain isolated in 1997 and has a very similar structure to other plasmids of the same lineage isolated a decade later (i.e., plasmid pMRSN3527-6, GenBank accession number NZ_CM003318.1). Additionally, plasmid p4ABAYE (GenBank accession number NC_010403.1), described in 2001, shared 98% sequence identity with pMRSN58-2.7 (GenBank accession number NZ_CM003316.1), isolated in 2013. Members of LN_19 are almost identical. The oldest member of the lineage was isolated in 2001 and the most recent in 2010 (**Supplementary Table S1**).

The *A. baumannii* plasmid sequences deposited in NCBI have increased since we last performed the analyses. On April 28, 2020, this database embraced the complete sequence of 422 A baumannii plasmids. To make a rapid evaluation of the prevalence of plasmid lineages LN_1, LN_2, LN_3A, LN_3B, and LN_4, we performed BLASTn on all members of these lineages against the new database. Using this strategy 30 new plasmids were incorporated in LN_1, 23 in LN_2, 12 in LN_3A+3B, and finally, 10 new plasmids were included in LN_4. Now, these lineages contain 38.4% of the *A baumannii* plasmids. However, we must say that the only way to identify all new members of the plasmid lineages is by reconstructing the networks with the rules mentioned above. These observations confirm that a few plasmid lineages encompass most of *A. baumannii* plasmids.

Plasmid Lineage Gene Composition

Comparisons of all members of a particular plasmid lineage with their representative plasmid show that the genome core of a plasmid lineage includes genes that are not involved in plasmid maintenance functions (the backbone) (**Figure 1** and **Supplementary Figures S2–S12**).

To obtain a general picture of the gene composition of our plasmid collection, we assigned a functional class (COG) to each of the protein products encoded by these plasmids. This analysis showed that these proteins fall within 23 functional classes; however, we were unable to assign a functional class (not in COG) to 74.15% of the proteins (**Figure 2**). In total, 3.53% of the encoded proteins have only a general function prediction (class R), and 3.5% are classified within class S (function unknown). Nevertheless, these 2497 uncharacterized or poorly characterized proteins were grouped in 242 orthologous groups [Remained Orthologous Groups (ROGs)] (Taboada et al., 2010). Therefore, it is not possible to predict whether some of these hypothetical proteins play a role in the nosocomial setting. However, given that the genes encoding these proteins are highly conserved within each plasmid lineage; the general structure of plasmids belonging to each one of the different lineages is stable during time and that the plasmids replicate in very different genetic backgrounds (even in different species), we suggest that these genes may play a role in reducing the fitness cost for the host to maintain the plasmids, thereby improving the favorability of the plasmids as parasite molecules.

We also found, as expected, a set of genes encoding proteins that are typically associated with plasmids: 7.28% of the proteins fall under class L (replication, recombination and repair), which includes replication initiation proteins, transposases, site-specific recombinases, and other proteins involved in recombination. Additionally, 1.31% of the proteins belong to class V (defense mechanisms), which includes proteins involved in plasmid stability (toxin-antitoxin modules) and restriction modification and proteins conferring antibiotic resistance. In the following sections, we will describe genes that play a crucial role in plasmid maintenance and that are usually associated with plasmid functions (**Figure 2**).

Classification of New Replication Initiation Protein (Rep) Genes

An absolute requirement for the survival of a plasmid is the presence of a replication module. These modules consist of an origin of replication, one gene encoding a replication initiator (Rep gene) and the factors and DNA sites involved in regulation of the expression of this gene, which is located near the Rep gene (del Solar et al., 1998). Our bioinformatic analysis indicates that from the 173 plasmids in our collection, 143 had an intact Rep gene and 13 plasmids had Rep pseudogenes, because we found on them premature stop codons or frameshifts generated, probably, during the sequencing and/or assembly processes. Nevertheless, in 27 plasmids, we could not find a Rep protein by annotation or BLAST searches; thus, as already noted by other authors, an experimental approach is needed to identify such replication regions (Lean and Yeo, 2017).

Rep genes of *A. baumannii* plasmids have been mainly identified by bioinformatic analyses, which have indicated that these proteins can be classified into five different categories: the most common Rep proteins belong to the Rep_3 superfamily (Pfam:01051) and are usually annotated as RepB. The next most frequent Rep proteins are those annotated as *replicases*, and these proteins have two distinctive domains: one is a replicase domain (pfam:03090), and the other is an alpha-helical domain that is also present at the C termini of primases (PriCT_1 superfamily). Other *A. baumannii* plasmids have Rep proteins belonging to the Rep_1 superfamily (Pfam01446). Several plasmids have a protein with a helix-turn-helix (HTH) domain annotated as a replication protein, and finally, one plasmid has an initiator protein classified as belonging to the RepC superfamily (Pfam:06504). Recently, the functionality of some of these replication regions was tested experimentally (Salto et al., 2018). Nevertheless, as mentioned above, many plasmids do not have an identifiable Rep protein. **Figure 3** and **Supplementary Figure S13** show the phylogenies of the replicases with the most members, separated by the domains identified by Pfam (Rep3 and replicase-PriCT). Some proteins were not included because either these proteins did not have

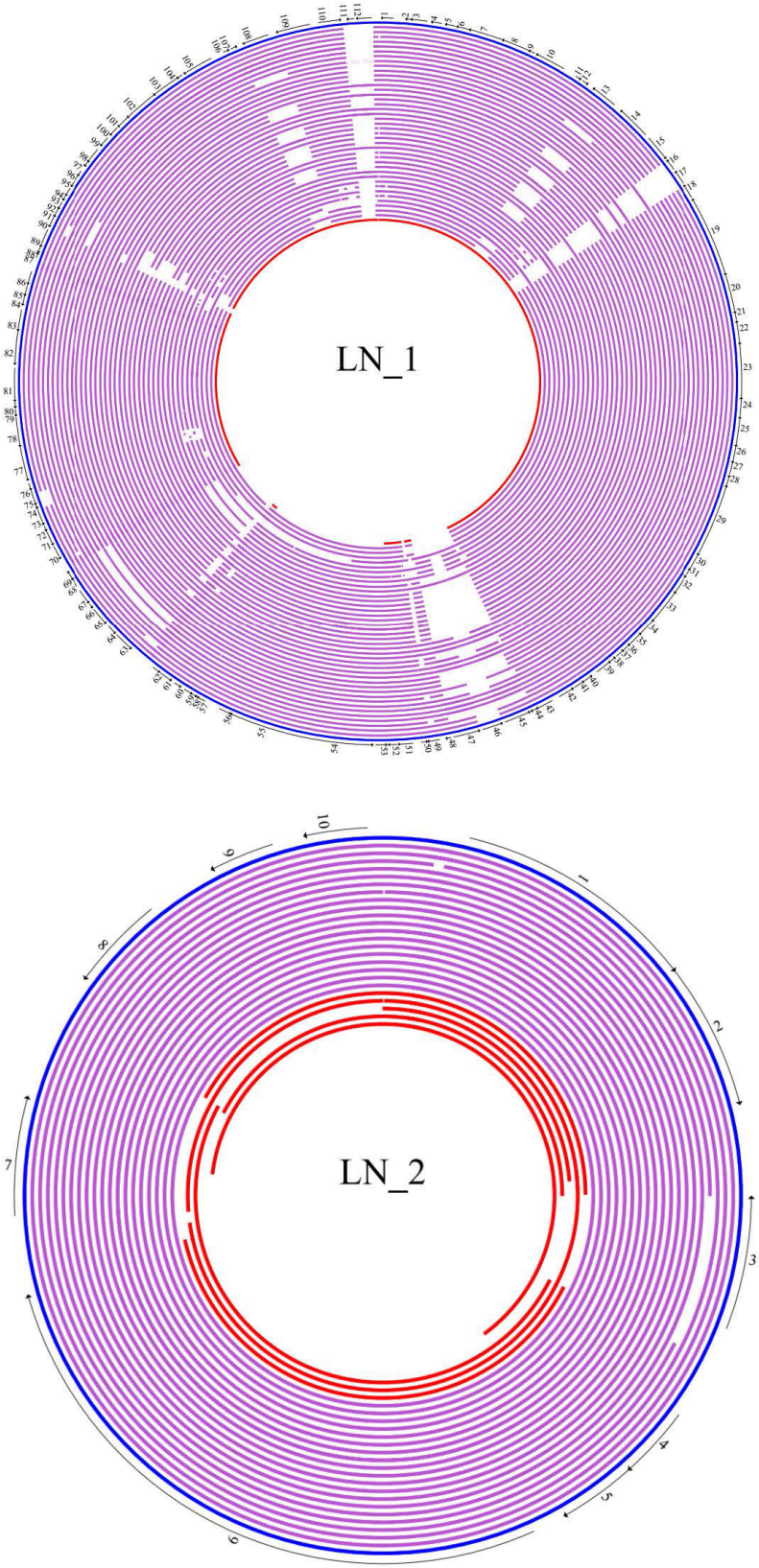

FIGURE 1 | Continued

FIGURE 1 | Cicular map of members of plasmid lineage LN_1 and linear map of members of LN_2. Plasmid sequences of each lineage were compared with its reference with BLASTn and mapped using GenVision a component of DNASTAR's Lasergene Core Suite. The genes core are in bold letters. The representative plasmid of LN_1 is plasmid AB34299 (blue ring) and numbers around this plasmid indicate the position of the following genes: **1. Nuclease; 2. Hypothetical protein; 3. Hypothetical protein; 4. Hypothetical protein; 5. Hypothetical protein; 6. Hypothetical protein; 7. Zeta-antitoxin; 8. Zeta-Toxin; 9. Hypothetical protein; 10. Plasmid replicase; 11. Hypothetical protein**; 12. Hypothetical protein; 13. Hypothetical protein; 14. DNA polymerase; 15. Hypothetical protein; 16. ISAba1; 17. ISAba1; **18. Transglycosylase; 19. Conjugal protein TraG; 20. Conjugal protein TraH; 21. Hypothetical protein; 22. Conjugal protein TraF; 23. Conjugal protein TraN; 24. Conjugal protein TrbC; 25. Conjugal protein TraU; 26. Conjugal protein TraW; 27. Peptidase; 28. Hypothetical protein; 29. Conjugal protein TraC; 30. Conjugal protein Tra; 31. Hypothetical protein; 32. Protein-disulfide isomerase; 33. Conjugal protein TraB; 34. Conjugal protein TraK; 35. Conjugal protein TraE; 36. Conjugal protein TraL; 37. Hypothetical protein; 38. Hypothetical protein; 39. Murein transglycosylase; 40. Hypothetical protein; 41. Hypothetical protein; 42. Resolvase; 43. Hypothetical protein**; 44. Hypothetical protein; 45. ISAba125; 46. Aminoglycoside phosphotransferase; 47. ISAba125; 48. Hypothetical protein; 49. Hypothetical protein; 50. Hypothetical protein; **51. Hypothetical protein**; 52. Hypothetical protein; 53. Hypothetical protein; 54. Relaxase MOBF; 55. Type IV secretion system protein VirD4; 56. Hypothetical protein; 57. Hypothetical protein; 58. Hypothetical protein; 59. Hypothetical protein; 60. Hypothetical protein; 61. Molecular chaperone DnaJ; 62. Hypothetical protein; 63. Hypothetical protein; 64. Hypothetical protein; 65. Hypothetical protein; 66. Hypothetical protein; 67. Hypothetical protein; 68. Addiction module toxin; 69. DNA-binding protein; **70. Hypothetical protein; 71. Hypothetical protein;** 72. Hypothetical protein; 73. Hypothetical protein; **74. Hypothetical protein**; 75. Hypothetical protein; 76. Hypothetical protein; 77. Hypothetical protein; **78. Toxic anion resistance protein TelA; 79. Hypothetical protein; 80. Hypothetical protein; 81. Hypothetical protein; 82. ParB family partition protein; 83. ParA family protein; 84. Hypothetical protein; 85. Hypothetical protein; 86. Hypothetical protein; 87. Hypothetical protein; 88. Hypothetical protein**; 89. Hypothetical protein; 90. Hypothetical; protein; 91. Hypothetical protein; 92. Hypothetical protein; 93. Hypothetical protein; 94. DNA-binding protein; 95. Nuclease; **96. Hypothetical protein; 97. Hypothetical protein; 98. Hypothetical protein; 99. Hypothetical protein; 100. Hypothetical protein; 101. Hypothetical protein; 102. Zeta-antitoxin; 103. Zeta-toxin; 104. Hypothetical protein; 105. Hypothetical protein; 106. Hypothetical protein; 107. Hypothetical protein**; 108. Hypothetical protein; **109. DNA polymerase**; 110. Hypothetical protein; 111. Transposase; 112. Transposase. Purple rings: Purple rings: from outside to inside: pNaval18-74, p2ABTCDC0715, pAC30c, pAba10042b,pAba9102a, pAba7847b, pACICU2, ABKp1, p1ABST2, pNaval81-67, pOIFC143-70, pIS123-67, pABUH1-74, p1AB5075, pAB04-2, plasmid YU-R612, pAba3207b, pCMCVTAb2-Ab4, plasmid CMC-CR-MDR-Ab66, plasmid KAB01, plasmid KAB02, plasmidKAB03, plasmid KAB04, plasmid KAB05, plasmid KAB06, pSSA12_1, pSSMA17_1,pJBA13_1, p15A34_1, pUSA2_1, pUSA15_1, pA85-3, pCS01A, pCS01B, pCR17A,pCR17B, pAba7835b, plasmid KAB07, plasmid KAB08, p15A5_1. Additional orphan plasmid is in red ring: pAC29b. LN_2. The representative plasmid of lineage LN_2 is pPKAB07 (blue bar). Numbers along this bar indicate the position of genes: **1. RepB family plasmid replication initiator protein; 2. DNA-binding protein**; 3. Hypothetical protein; 4. Toxin-Antitoxin system spITA (COG3514); 5 Toxin-Antitoxin system spITA (DUF497); 6. TonB dependent receptor; 7. Hypothetical protein; 8. Hypothetical protein; 9. Hypothetical protein; 10. Hypothetical protein. Purple bars: from top to bottom: p1ABTCDC0715, pAC12, pAC30a, p2ABAYE, pAB0057, p1BJAB0868, pCanadaBC5-8.7, pABUH6a-8.8, pMRSN7339-8.7, pMRSN58-8.7, pAB0057, plasmid_2 AB34299, p2AB5075, pAC29a, pA1-1, p15A5_2, pSSA12_2, pA85-2, pAB5075. Additional orphan plasmids are in red bars: from top to bottom: pAB2, p1ABST78, pORAB01-3, p MEX11594, pYU-R612.

any Pfam domain assigned in the database or there were not enough members to perform comparisons, as in the extreme case of the RepC domain, with only one protein assigned to this domain(El-Gebali et al., 2019).

In 2010, Bertini and coworkers designed a classification system for the *A. baumannii* plasmids based on the nucleotide identity of the Rep genes (Bertini et al., 2010). Rep genes that shared at least 74% nucleotide identity were pooled in the same group. With this scheme, the authors identified 19 homology groups (GR1 to GR19). Subsequently, Lean and Yeo, studying A. *baumannii* plasmids of less than 10 kb, proposed a new group based on Rep phylogenetic analyses: GR20, which is closely related to GR2; however, the members of this group form a clear separate clade (Lean and Yeo, 2017). Recently, Cameranesi and collaborators analyzed *A. baumannii* plasmids from Argentina and determined that some Rep genes of these plasmids required the formation of three additional groups: GR21, GR22 and GR23 (Cameranesi et al., 2018). However, the analysis of the genes annotated as Rep proteins from our plasmid collection showed that the current classification system was not sufficient to include all the Rep proteins. Therefore, by following the scheme proposed by Bertini and coworkers, ten additional groups were constructed (GR24-GR33). These replication gene groups can be visualized as a network in which one gene encoding a replication protein is part of a group if its DNA sequence shares at least 74% identity and 90% coverage with another member of the same group. For each new Rep group (GR), we chose the most interconnected member as the representative sequence of the group. However, we identified some unusual Rep proteins that showed nucleotide sequence identity higher than 74% with members of two different groups. This inconsistency was provoked because some authors named new replication homology groups, using a different set of rules of those originally proposed by Bertini and coworkers. Examples, the representative protein of GR23 is identical to that of GR8_1 proposed by Bertini and coworkers or the representative members of groups GR2 and GR20 have a DNA sequence identity higher than 74%. (Fondi et al., 2010; Cameranesi et al., 2018). In these cases, we assigned the unusual protein to the group with which this element shared the highest nucleotide identity (**Supplementary Table S2**). The assignations of all the replication proteins encoded in our plasmid set are listed in **Supplementary Table S1**. Seven of the new groups harbor DNA initiator proteins of the Rep_3 family (GR26, GR27, GR28, GR29, GR30, GR31, GR32); two of these groups are composed of proteins of the Replicase_PriCT family (GR25 and GR32), but the representative member of GR25 has an HTH_29 additional conserved domain (Pfam13551) and finally, one plasmid carries a Rep protein of the RepC family (GR33). We were incapable of identifying a gene encoding a Rep protein in three plasmid lineages (LN_4, LN_7 and LN_18) and in 14 orphan plasmids. On the other hand, four plasmid lineages, namely, LN_2, LN_11, LN_13 and LN_20, exhibited the same organization in their replication modules. This module consists of a bicistronic operon, in which the first gene encodes an initiator protein of the Rep_3 family (or RepB), and the second gene of the operon encodes a protein with an HTH motif that on

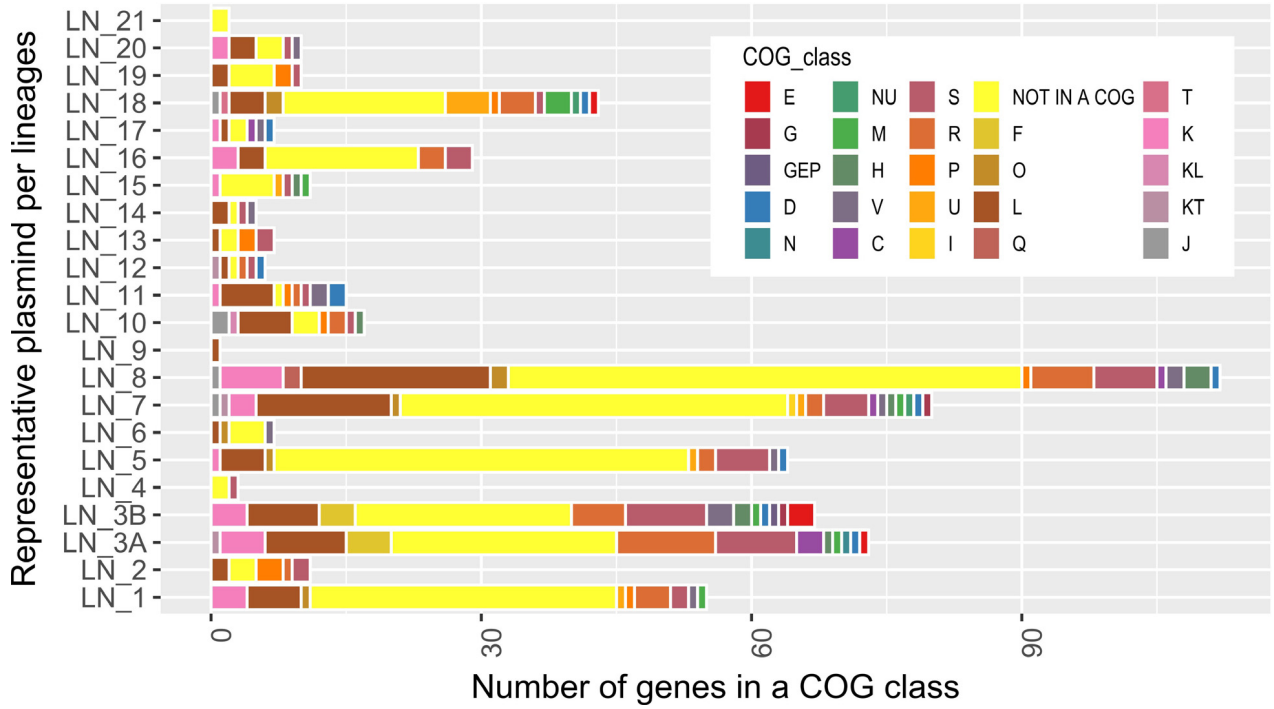

FIGURE 2 | Number of genes assigned to a functional class (COG) present in the representative plasmid of each lineage. Classes: CO, energy production and conversion, posttranslational modification, protein turnover, chaperones. DJ, cell cycle control, cell division, chromosome partitioning, translation, ribosomal structure and biogenesis. Q, secondary metabolites biosynthesis, transport and catabolism I, Lipid transport and metabolism. GEP, carbohydrate transport and metabolism, amino acid transport and metabolism, inorganic ion transport and metabolism. KT, transcription, signal transduction mechanisms. NU, cell motility, intracellular trafficking, secretion, and vesicular transport. G, carbohydrate transport and metabolism. KL, transcription, replication, recombination and repair. J, translation, ribosomal structure and biogenesis. T, signal transduction mechanisms. E, amino acid transport and metabolism. D, cell cycle control, cell division, chromosome partitioning. H, coenzyme transport and metabolism. F, nucleotide transport and metabolism. C, energy production and conversion. M, cell wall/membrane/envelope biogenesis. U, intracellular trafficking, secretion, and vesicular transport. O, posttranslational modification, protein turnover, chaperones. P, inorganic ion transport and metabolism. V, defense mechanisms. K, transcription. S, function unknown. R, general function prediction only. L, Replication, recombination and repair. NOT IN A COG, COG not defined.

some occasions has been wrongly annotated as a putative Rep protein, for example, in the homology group GR17. We traced the error source to an obvious mistake in GenBank: plasmid pAB1 (GenBank accession number CP000522.1) carries a gene annotated as encoding a DNA replication protein (protein_id ABO13850.1) which is precisely the representative member of GR17. This putative DNA replication protein carries an HTH_17 conserved domain (Pfam12728). However, a BLAST search indicates that the gene upstream to that encoding the HTH-carrying protein encodes a protein belonging to the Rep_3 superfamily (protein_id ABO13860.1) which is identical to other *A. baumannii* replication proteins. Unfortunately, this gene is annotated as encoding a hypothetical protein. To facilitate future work, Rep proteins sequences and the genes that codify them are listed in **Supplementary Materials S1**, **S2**. Be careful: these lists still include the representative member of GR17 described above.

Iterons in the New GR Replication Homology Groups

It has been shown that *iterons*, which are small repetitive DNA sequences located near the Rep gene, usually in tandem, play a crucial role in the control of plasmid replication in many plasmids (Chattoraj, 2000; Wegrzyn et al., 2016). These sequences have been bioinformatically identified in some *A. baumannii* plasmids; therefore, we searched for the presence of these sequences in the representative Rep genes and their surrounding sequences in each of the new GR groups (GR24-GR33) (Lean and Yeo, 2017; Salto et al., 2018). We could identify such tandem repeats near the initial codon of the Rep protein in six of these groups (GR24, GR26-GR30). The putative iterons of each one of the new groups are shown in **Supplementary Table S3**. Interestingly, in these cases, we also identified a region rich in A+T near these tandem repeats, which is a typical characteristic of plasmid replication origins. Of course, these presumptions must be tested in the laboratory. In contrast, GR25, GR31, GR32, and GR33 do not have iterons, at least not near the Rep gene. The first two Rep genes belong to the Rep_PriCT family and GR33 is the only member of the RepC family in our collection. Plasmid pD36-4 is a bireplicon that encodes two Rep proteins of the Rep_3 superfamily: RepA1 (WP_000140303.1) (GR31) and RepA2 (protein_id WP_000786839.1) (Hamidian and Hall, 2018a). The RepA2 gene is preceded by three copies of a 19 bp iteron

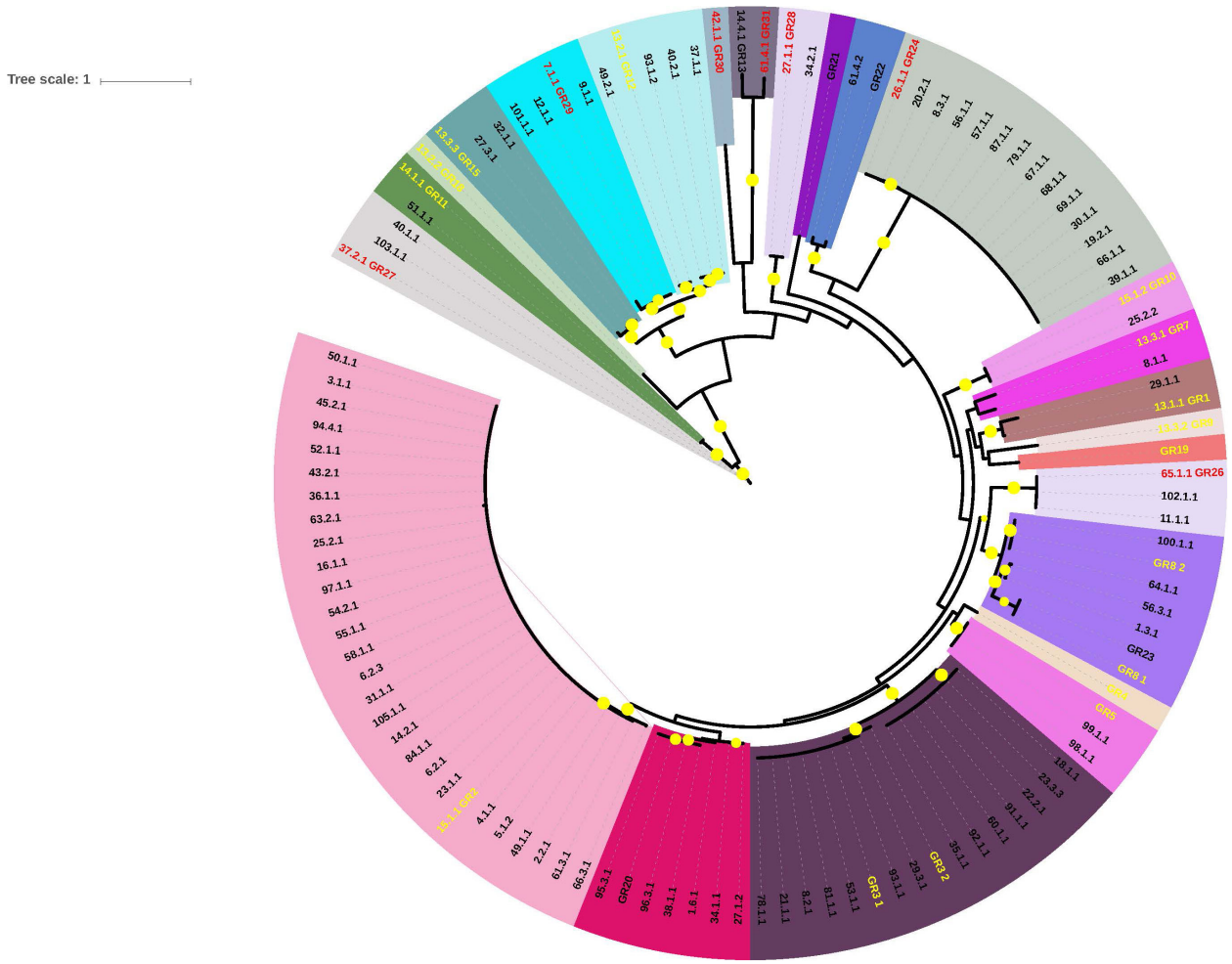

FIGURE 3 | Phylogenetic tree of genes encoding replicase proteins belonging to the Rep_3 family. Gene codons were aligned guided by protein alignments. In this figure we are using the replicase_ID numbers listed in **Supplementary Table S2**. Each color embrace members of one clade. Names with yellow letters indicate the reference genes used by Bertini et al. (2010) to construct GR homology groups. Names with red letters show the reference genes used by us to construct the new GR homology groups. Bootstrap values higher than 70% are marked in the figure with yellow circles.

sequence, but surprisingly, RepA1 does not possess iterons at least 500 bp upstream of the initiation codon, 500 bp downstream of the stop codon or within the Rep coding region, suggesting that this protein is no longer responsible for pD36-4 replication(Hamidian and Hall, a).

Plasmid Incompatibility and Initiator Proteins

Plasmid incompatibility has been defined as the inability of two replicons to coexist in the same cell line. This phenomenon occurs when some elements of the replication or partitioning machineries of a plasmid interfere with the maintenance functions of a second plasmid (Novick, 1987; Austin and Nordström, 1990). Thus, different plasmids that are stably maintained in the same bacterial cell belong, by definition, to different incompatibility groups. On the other hand, plasmids that are mutually incompatible are classified within the same incompatibility group and, very frequently, are phylogenetically closely related.

An inspection of the initiator genes in each of the plasmid lineages with only one Rep gene shows that all members of the same plasmid lineage share a replication initiator protein, classified within the same Rep homology group as defined by Bertini and coworkers (Bertini et al., 2010). For example, Rep proteins of lineage 1 belong to Rep group GR6; Rep proteins of lineage 2 belong to GR2; Rep proteins of lineage 3 belong to GR24, and those of LN_5 are classified within GR25 (**Supplementary Table S1**). In many cases, Rep proteins of the same GR group have amino acid sequences that are identical or almost identical: for example, all Rep proteins within LN_2 or LN_3A are identical, and those of LN_1 share 99.1% sequence identity among each other.

Plasmid lineages (LN_8, LN_10, LN_16) share Rep proteins of the same GR homology group (GR3); however, a protein

alignment performed with Clustal Omega indicates that Rep proteins of LN_8 and LN_10 are almost identical (>99.6%). Differences between LN_8 and LN_10 and LN16 is 80.2%. In our collection, members of these plasmid lineages are never located in the same bacterial isolate; however, differences in the sequences of Rep proteins between these two groups could be significant enough to represent two incompatibility groups. Recently, Blackwell and Hall (2019) showed that plasmids pS32-1 and pS21-a are compatible and that these plasmids contain Rep proteins of the Rep_3 superfamily. Interestingly, these proteins share a protein sequence identity of 85.4% (Blackwell and Hall, 2019). Nevertheless, an experimental approach is needed to resolve these problems. Our analysis indicates that plasmids of the same isolate belong to different plasmid lineages, with two exceptions, namely, isolates CR17 and CS01, which are almost identical in sequence. Each isolate possesses three plasmids, and two of the plasmids in each strain belong to LN_1; however, we were unable to identify complete Rep genes in these four plasmids; we could identify only truncated Rep genes or pseudogenes. Therefore, we could not elucidate the mechanisms via which these plasmids replicate or coexist in the same isolates. Taken together, these observations suggest that members of a plasmid lineage belong to the same incompatibility group.

Bi- and Trireplicons

It has been previously observed that some *A. baumannii* plasmids contain more than one gene encoding a Rep protein. In fact, there are some examples of such plasmids in our collection: 5 plasmids possess two Rep genes, and one plasmid, p3ABSDF, contains 3 Rep genes (**Supplementary Table S4**). Each one of the Rep genes residing in the same plasmid belongs to a different Rep group. Some of the isolates that have bi- or trireplicons also contain other companion plasmids; these companion plasmids always include replication modules belonging to different Rep groups, between each other and with those present in the multireplicon plasmid. The French isolate SDF is an extreme example present in our collection. This isolate has three plasmids: p1ABSDF, p2ABSDF and p3ABSDF. The first plasmid possesses a replication module classified within Rep group GR1. The second plasmid, p2ABSDF, has two replication modules, one belonging to GR12 and the other to GR18. The third plasmid has 3 replication modules that belong to different Rep groups: GR7, GR9 and GR15. All the GR homology groups present in each isolate differ, preventing potential functional interference between the groups. These observations reinforce our hypothesis that each plasmid lineage belongs to a different incompatibility group and also suggest that these plasmids are the products of ancient plasmid cointegrations.

We also identified a plasmid lineage, LN_3 with a Rep protein belonging to homology group GR24. Members of this lineage contain a large set of phage-related genes, including several that could be implicated in replication, such as a DNA primase, a DNA helicase, a DNA ligase, the catalytic domain of DNA polymerase III (subunit α), and exonucleases, as already observed by Huang and coworkers (Huang et al., 2014). Plasmids that are capable of using phage-related proteins in replication can be considered bireplicons.

Partitioning Modules

Plasmids of high molecular weight and low copy number require an active segregation machinery to ensure that newly replicated plasmids are adequately segregated into the daughter cells. To date, three different active segregation machinery types have been identified, all of which consist of an NTPase, a centromere-like binding protein and at least one centromere-like sequence. These segregation machineries have been classified into three types according to their NTPase proteins: type I, which has a Walker-type ATPase (ParA); type II, which contains actin-like ATPases (ParM); and type III, which possesses a GTPase similar to tubulin (TubZ) (Baxter and Funnell, 2014). However, by far, the most common segregation machinery is that belonging to type I. This type consists of three different elements: ParA, a Walker-type ATPase; ParB, which is a centromere-like binding protein; and a DNA centromere-like site (*parS*). These systems are usually organized in an operon in which the first gene is *parA*, followed by *parB*, and the *parS* site is usually located near the *parA/parB* genes. Generally, plasmids that use this segregation system possess only one copy of the operon (Bignell and Thomas, 2001).

Of the *A. baumannii* plasmids studied here, lines LN_1, LN_5, LN_7 and LN_8 have *parA/parB* genes in the classic conformation, but members of LN_8 contain duplicates of these genes. In contrast, other lineages possess incomplete *parA/parB* systems: LN_3 members have one copy of *parA* and two copies of *parB*; LN_11 contains only one *parA* gene per plasmid and LN_18 members contain one *parB* copy. Interestingly, all members of LN_8 also encode a ParM-like protein, suggesting that these plasmids may possess a second segregation system belonging to type II. These observations revealed an extensive diversity of plasmid segregation systems in *A. baumannii* (**Supplementary Table S1**).

Toxin-Antitoxin Modules

Plasmids have developed several genetic modules to ensure their persistence within a bacterial population, and some of these modules are classified as toxin-antitoxin (TA) modules. These modules consist of two genes: one encoding a toxin and the other its cognate antitoxin. Toxins are more stable than antitoxins; therefore, cells that lose a plasmid encoding one of these modules are eventually eliminated from the population (Hayes and Van Melderen, 2011; Unterholzner et al., 2013). The presence of these modules on plasmids not only ensures the persistence of the plasmids within a cell line but may also play a role in bacterial virulence (Lobato-Márquez et al., 2016). TA modules been previously described in *A. baumannii* plasmids; therefore, we searched for the presence of these modules in our 173 plasmids (Jurenaite et al., 2013; Sužiedėlienė et al., 2016; Armalytė et al., 2018). We determined that 108 of them have TA modules belonging to nine different classes. Eight of these modules were TA modules of type II: ZetaTA (43.5%), SplTA (30.5%) and HigB/A (11.1%), and other TA modules that were less well represented (13.9%, in total), including YafQ/RelB, RelB/E, HicAB, HipA/B, and Phd/YoeB. Four plasmids have the

TA module AbiEii/AbiGii (type IV). Plasmids with TA modules exhibit the general tendency to have one per plasmid, with one exception: the orphan plasmid p3ABAYE has three different TA systems, namely, HigB/A, HipA/B, and RelB/E. Plasmids with the same TA module, in general, do not coexist. We have two isolates, namely, CR17 and CS01, that each possess one plasmid of the same lineage and with the same TA modules (**Supplementary Figure S14** and **Supplementary Table S5**).

Plasmid-carried restriction-modification modules play a role in plasmid stabilization via postsegregational killing (Kulakauskas et al., 1995) therefore, we searched for these modules in the plasmid collection, and only 7.9% of the plasmids harbor these modules. We showed that only five members of LN_1 and two plasmids of LN_3B have these modules. Three orphan plasmids, namely, pOIFC032-101, p2ABSDF and p3ABSDF, also have restriction-modification modules. Some plasmids, such as those belonging to LN_8 and the orphan plasmid pHWBA8_1, encode only for the DNA methyltransferase. These results suggest that some members of LN_1 and LN_3B acquired restriction-modification modules after the origination and diversification of the lineages.

Conjugation Modules

Conjugation is probably the most efficient process for dissemination of plasmids among strains of the same species or even to not closely related species. This process requires two gene sets: one involved in mating pair formation, which encompasses all genes required for the synthesis of a specialized type 4 secretion system that is essential for establishment of contacts between donor and receptor cells. The second gene set encodes products required for DNA processing and replication. Plasmids with these two functional gene sets are self-transmissible. However, other plasmids, containing only a transfer origin (*oriT*), a relaxase gene and some genes encoding nicking accessory proteins, require for mobilization of their proteins a specialized type 4 secretion system encoded by a second (helper) plasmid. These plasmids are known as mobilizable plasmids (Smillie et al., 2010; Cabezón et al., 2015). We performed a bioinformatic search for genes involved in conjugation in our plasmid collection, and the results are summarized in **Supplementary Table S5**. We discovered that only two plasmid lineages, namely, LN_1 and LN_5, have large sets of conjugation genes (>10 genes), but only members of LN_1 have been experimentally shown to be capable of conjugation (Di Venanzio et al., 2019). One of the 39 orphan plasmids, pKBN10P02143, has a large set of conjugation genes, suggesting that this plasmid is also conjugative. We also found some plasmids that have a small set of six conjugation genes but not a gene encoding a relaxase, such as members of lineages LN_7 and LN_8, suggesting that in the mobilization capacity was lost during evolution.

Eight of the 21 plasmid lineages identified in this work have the potential to be mobilizable, considering that these lineages have relaxase genes and their cognate *oriT* sequences. Six of these plasmid lineages (LN_12, LN_14, LN_15, LN_17, LN_18 and LN_3B) have relaxase genes belonging to the MOB_Q family, and all of these genes are closely related to other relaxases described only for *A. baumannii* plasmids (Salto et al., 2018). Lineages LN_1 and LN_5 have relaxase genes of the MOB_F family, and members of LN_4 possess a relaxase gene of the MOB_H family. Thirteen orphan plasmids have MOB_Q relaxase genes and only one relaxase gene of the MOB_P family. Notably, 14 plasmid lineages do not have relaxase genes; however, some of these lineages are dispersed throughout the A baumannii phylogenetic tree constructed with ribosomal genes not containing recombination signals. However, it has been shown that some *Staphylococcus aureus* plasmids, even in the absence of a relaxase and relaxase accessory genes, have sequences that mimic *oriT* sequences and that can be used for mobilization when they coexist with a conjugative plasmid that encodes Mob proteins able to recognize these oriT sequences (O'Brien et al., 2015a,b). Recently, Blackwell and Hall (2019) showed that the conjugative plasmid (pAb-G7-2) was capable to mobilize plasmid pS32-1, which lacks Mob encoding genes, through a relaxase *in trans* mechanism (Blackwell and Hall, 2019). Making sequence comparisons, these authors suggest that plasmid pS32-1 has a 32 pb DNA sequence that closely matches in sequence and organization the *oriT* of plasmid R388, an IncW plasmid whose *oriT* has been experimentally dissected. Blackwell and Hall also showed that the putative *oriT* and their adjacent sequences are present in other *A. baumannii* plasmids (Blackwell and Hall, 2019). To expand these observations, we search for the presence of these sequences in our plasmid collection set and here we show that they are present in all members of LN_2, LN_11, LN_19, LN_20 and some other plasmid, including a couple of orphans, indicating that potentially this mechanism is the responsible to disperse this plasmid lineages through different *A. baumannii* clades. DNA alignment of the putative *oriT* sequences located in these plasmids is shown in **Figure 4**. Nevertheless, these observations in conjunction also suggest that other plasmid transmission mechanisms that are not dependent on type IV secretion systems, such as transduction, transformation or outer membrane vesicles may play an important role in the spread of plasmids between *A. baumannii* populations (Rumbo et al., 2011; Chatterjee et al., 2017).

All *A. baumannii* strains studied here contain in their chromosomes genes encoding a type VI secretion system (T6SS) that is used to eliminate nonkin bacteria (Weber et al., 2013). An essential requirement for conjugation and T6SS functioning requires a tight cell-to-cell contact, and for this reason, conjugation can only take place when the T6SS is repressed, otherwise, the receptors for conjugation will be killed. Weber et al. (2015) demonstrated that large *A. baumannii* conjugative plasmids, all belonging to LN_1, encode two proteins TetR1 and TetR2 that repress the expression of the T6SS system and in this way promoting the dissemination not only of LN_1 plasmids but also of those mobilizable plasmids that coexist with them (Weber et al., 2015; Di Venanzio et al., 2019). These observations explain why LN_1 plasmids are widely distributed along many *A. baumannii* strains.

Insertion Sequences

IS elements and transposons are mobile genetic elements that can move from one location to another on the same replicon or between replicons of the same cell, but if linked

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pS32-1                MG954378.1        ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
pAB5075        LN_2.  NZ_JHUI01000005.1  ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
pA85-2         LN_2   NZ_CP021786.1     ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
pSSA12-2       LN_2   NZ_CP020576.1     ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
unnamed2       LN_2   NZ_CP014217.1     ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
pA1-1.         LN_2   NZ_CP010782.1     ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
pAC29a         LN_2   NZ_CP008850.1     ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
p2AB5075.      LN_2   NZ_CP008708.1     ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
pPKAB07        LN_2   NZ_CP006964.1     ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
pAB0057        LN_2   NZ_CM003909.1     ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
unnamed1       LN_2   NZ_CM003741.1     ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
pMRSN58-8.7    LN_2   NZ_CM003317.1     ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
pMRSN7339-8.7  LN_2   NZ_CM003314.1     ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
pABUH6a-8.8.   LN_2   NZ_AYEX01000118.1 ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
pCanadaBC5-8.7 LN_2   NZ_AFDN01000003.1 ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
p1ABST78.      LN_2   NZ_AEOZ01000236.1 ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
p1BJAB0868     LN_2   NC_021730.1       ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
p2ABAYE        LN_2.  NC_010402.1       ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
unnamed2       LN_2   CP014293.1        ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
pAC30a         LN_2   CP007578.1        ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
pAC12          LN_2.  CP007550.1        ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
p1ABTCDC0715   LN_2   CP002523.1        ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
p15A5_2.       LN_2   NZ_CP020575.1     GATTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
pAB2.          LN_2.  CP000523.1        ATTTTTTTGTCACACACCACGCATCTAACGATGAAACCCTTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
p2ABST2.       LN_11  NZ_AEOY01000096.1 ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
pACICU1.       LN_11  NC_010605.1       ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
pCS01C         LN_13  NZ_HG977525.1     ATTTTTTTGTCACACACCACGCATCTAACGATGAAACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
pNaval17-13.   LN_19  NZ_AFDO01000021.1 ATTTTTTTGTCACACACCACGCATCTAAAGATGACAGCCCTCAAAACCTTACAGGATAAAGGTTTCAGCAATT
pNaval81-13.   LN_19  NZ_AFDB02000005.1 ATTTTTTTGTCACACACCACGCATCTAAAGATGACAGCCCTCAAAACCTTACAGGATAAAGGTTTCAGCAATT
pAba3207a      LN_20  NZ_CP015365.1     ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGTATAAGGGTTTTAGTATTT
pAba7835a      LN_20  NZ_CP033244.1     ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGTATAAGGGTTTTAGTAATT
pAba3207a      LN_20  NZ_CP015365.1     ATTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGTATAAGGGTTTTAGTAATT
unnamed2.      ORPH   NZ_CM003742.1     TTTTTTTTGTCACACACCACGCATCTAACGATGACACCCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
pD36-3.        ORPH   NZ_CP012955.1     ATTTTTTTGTCACACACCACGCATCTAAAGATGACAACCCTCAAAGCCTTACAGGATAAGGATTTCAGCGATT
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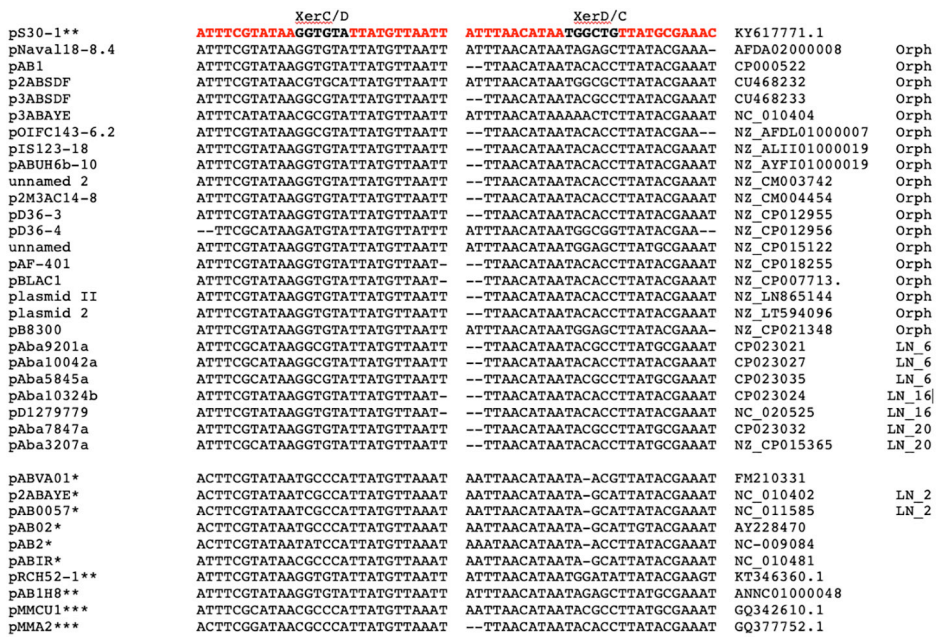
FIGURE 4 | DNA sequence alignment of *oriT* regions (73 bp) located in *A. baumannii* plasmids. At the left, plasmid names followed by the plasmid lineages and by their accession numbers. Letters in red are the nucleotides that show differences with the *oriT* of plasmid pS32.1 (at the top).

to other mobile elements such as plasmids or phages, these elements can be horizontally transmitted to other genomes (Siguier et al., 2014). These elements play an essential role in genome plasticity and gene expression and play a crucial role in bacterial pathogens because antibiotic resistance genes are frequently linked to these elements (Partridge et al., 2018). However, 47.3% of the plasmids in our collection do not have IS elements. The remaining plasmids analyzed here have at least one IS element of the 41 different IS elements identified in the collection (**Supplementary Table S6**). The most common IS elements were IS*Aba1* (13.1%) and IS*Aba125* (12.6%). The plasmid lineages exhibit contrasting features in terms of the number and diversity of IS elements: some plasmid lineages do not contain IS elements, such as LN_2 and LN_4. However, all the members of some lineages have IS elements. Some of these plasmids share the same IS elements or set of IS elements located in the same region (LN_10), while members of other lineages include different IS elements (i.e., LN_7). In conjunction, some lineages have members that lack IS elements; others have members with one IS; and the remaining include several IS elements of different kinds scattered along their DNA sequences. One of these lineages is LN_1. This lineage includes 42 members. Eight of these members do not possess IS elements; 22 members have only one element (the most frequent element being IS*Aba125*); and the remaining members have 2-4 IS elements. This observation clearly shows that IS elements are secondary acquisitions in the genomes of the members of this lineage. The plasmid lineages with a high number of IS elements and which exhibit high diversity in IS families are LN_8 and LN_7.

XerCD Recombinase and *Pdif* Sites

XerCD recombinases and their action sites (*dif* or XerC/D and XerD/C sites) have an important role resolving chromosome and plasmid dimers to monomers, but also in other site-specific reactions like the integration of the phage CTX at the *dif1* site of *Vibrio cholerae* chromosome I (Summers and Sherratt, 1988; Val et al., 2005). The presence of homologous *dif* sequences (*pdif*) has been found in many *A. baumannii* plasmids and they consist of stretches of 28 bp that contain the binding sites for the XerC and XerD recombinases (11 bp each) separated by a variable 6 bp linker. It has been proposed that these sites play a role in the mobilization of discrete DNA modules between *A. baumannii* replicons (D'Andrea et al., 2009; Blackwell and Hall, 2017). These modules have an important role in the dissemination of antibiotic resistance genes, since some of them embrace antibiotic-resistant genes like OXA-58 and OXA-24/40 (Poirel and Nordmann, 2006; Merino et al., 2010; Grosso et al., 2012), genes involved in tetracycline resistance (*tet39*), or the *msrE* and *mphE* macrolide resistance genes (Blackwell and Hall, 2017). We evaluated the presence of these sites in our plasmid set using as query the *pdif* sites of plasmid pS30-1 described by Blackwell and Hall (2017). Many plasmids of our collection possess at least one XerC/D site and others, but not necessarily the same plasmids, have one or several XerD/C sites, but only 15 plasmids have



	XerC/D	XerD/C		
pS30-1**	ATTTCGTATAAGGTGTATTATGTTAATT	ATTTAACATAATGGCTGTTATGCGAAAC	KY617771.1	
pNaval18-8.4	ATTTCGTATAAGGTGTATTATGTTAATT	ATTTAACATAATAGAGCTTATACGAAA-	AFDA02000008	Orph
pAB1	ATTTCGTATAAGGTGTATTATGTTAATT	--TTAACATAATACACCTTATACGAAAT	CP000522	Orph
p2ABSDF	ATTTCGTATAACGTGCATTATGTTAATT	ATTTAACATAATGGCGCTTATACGAAAT	CU468232	Orph
p3ABSDF	ATTTCGTATAAGGCGTATTATGTTAATT	--TTAACATAATACGCCTTATACGAAAT	CU468233	Orph
p3ABAYE	ATTTCATATAACGCGTATTATGTTAATT	ATTTAACATAAAAACTCTTATACGAAAT	NC_010404	Orph
pOIFC143-6.2	ATTTCGTATAAGGCGTATTATGTTAATT	--TTAACATAATACACCTTATACGAA--	NZ_AFDL01000007	Orph
pIS123-18	ATTTCGTATAAGGTGTATTATGTTAATT	--TTAACATAATACACCTTATACGAAAT	NZ_ALII01000019	Orph
pABUH6b-10	ATTTCGTATAAGGTGTATTATGTTAATT	--TTAACATAATACACCTTATACGAAAT	NZ_AYFI01000019	Orph
unnamed 2	ATTTCGTATAAGGTGTATTATGTTAATT	--TTAACATAATACACCTTATACGAAAT	NZ_CM003742	Orph
p2M3AC14-8	ATTTCGTATAAGGTGTATTATGTTAATT	--TTAACATAATACACCTTATACGAAAT	NZ_CM004454	Orph
pD36-3	ATTTCGTATAAGGTGTATTATGTTAATT	--TTAACATAATACACCTTATACGAAAT	NZ_CP012955	Orph
pD36-4	--TTCGCATAAGATGTATTATGTTATTT	ATTTAACATAATGGCGGTTATACGAA--	NZ_CP012956	Orph
unnamed	ATTTCGTATAAGGTGTATTATGTTAATT	ATTTAACATAATGGAGCTTATGCGAAAT	NZ_CP015122	Orph
pAF-401	ATTTCGTATAAGGTGTATTATGTTAAT-	--TTAACATAATACACCTTATACGAAAT	NZ_CP018255	Orph
pBLAC1	ATTTCGTATAAGGTGTATTATGTTAAT-	--TTAACATAATACACCTTATACGAAAT	NZ_CP007713.	Orph
plasmid II	ATTTCGTATAAGGTGTATTATGTTAATT	--TTAACATAATACACCTTATACGAAAT	NZ_LN865144	Orph
plasmid 2	ATTTCGTATAAGGTGTATTATGTTAATT	--TTAACATAATACACCTTATACGAAAT	NZ_LT594096	Orph
pB8300	ATTTCGTATAAGGTGTATTATGTTAATT	ATTTAACATAATGGAGCTTATACGAAA-	NZ_CP021348	Orph
pAba9201a	ATTTCGCATAAGGCGTATTATGTTAATT	--TTAACATAATACGCCTTATGCGAAAT	CP023021	LN_6
pAba10042a	ATTTCGCATAAGGCGTATTATGTTAATT	--TTAACATAATACACCTTATACGAAAT	CP023027	LN_6
pAba5845a	ATTTCGCATAAGGCGTATTATGTTAATT	--TTAACATAATACGCCTTATGCGAAAT	CP023035	LN_6
pAba10324b	ATTTCGTATAAGGTGTATTATGTTAAT-	--TTAACATAATACACCTTATACGAAAT	CP023024	LN_16
pD1279779	ATTTCGTATAAGGTGTATTATGTTAAT-	--TTAACATAATACACCTTATACGAAAT	NC_020525	LN_16
pAba7847a	ATTTCGTATAAGGTGTATTATGTTAATT	--TTAACATAATACGCCTTATGCGAAAT	CP023032	LN_20
pAba3207a	ATTTCGCATAAGGTGTATTATGTTAATT	--TTAACATAATACACCTTATGCGAAAT	NZ_CP015365	LN_20
pABVA01*	ACTTCGTATAATGCCCATTATGTTAAAT	AATTAACATAATA-ACGTTATACGAAAT	FM210331	
p2ABAYE*	ACTTCGTATAATCGCCATTATGTTAAAT	AATTAACATAATA-GCATTATACGAAAT	NC_010402	LN_2
pAB0057*	ACTTCGTATAATCGCCATTATGTTAAAT	AATTAACATAATA-GCATTATACGAAAT	NC_011585	LN_2
pAB02*	ACTTCGTATAATGCCCATTATGTTAAAT	AATTAACATAATA-GCATTGTACGAAAT	AY228470	
pAB2*	ACTTCGTATAATATCCATTATGTTAAAT	AAATAACATAATA-ACCTTATACGAAAT	NC-009084	
pABIR*	ATTTCGTATAACGCCCATTATGTTAAAT	AATTAACATAATA-GCATTATACGAAAT	NC_010481	
pRCH52-1**	ATTTCGTATAAGGTGTATTATGTTAATT	ATTTAACATAATGGATATTATACGAAGT	KT346360.1	
pAB1H8**	ATTTCGTATAAGGTGTATTATGTTAATT	ATTTAACATAATAGAGCTTATGCGAAAT	ANNC01000048	
pMMCU1***	ATTTCGCATAACGCCCATTATGTTAAAT	AATTAACATAATACGCCTTATGCGAAAT	GQ342610.1	
pMMA2***	ACTTCGGATAACGCCCATTATGTTAAAT	--TTAACATAATACACCTTATACGAAAT	GQ377752.1	

FIGURE 5 | Alignment of left *pdif* (XerC/D) and right *pdif* (XerC/D) plasmids sites (in red) and their flanking sequences. At the top sequences used as query. At the left, plasmid names followed by XerC/D and XerC/D sequences. Next, column list GenBank accession numbers followed by the lineage number. At the bottom and marked with asterisks are plasmid and *pdif* sites defined by other authors: D'Andrea et al. (2009); Merino et al. (2010), and Blackwell and Hall (2017).

matches with both sequences. The list of the plasmids possessing these sites and the DNA sequence alignment of these sites are shown in **Figure 5**. In this work, we analyzed the *pdif* modules with antibiotic resistance genes. This analysis revealed some of the gene modules described by other authors in new plasmids. For example, the module of plasmid pS30-1 carrying *tetR* and *tet39* genes and involved in tetracycline resistance is also present in the orphan plasmid pNaval18-8.4 (Blackwell and Hall, 2017). However, in plasmids of the Mexican isolates, we found two new *pdif* modules. One of them of 967bp contains an OXA-72 gene and was identified in the members of LN_6. The second was present in plasmids pAba7847a and pAba3207a of LN_20 and consists in a 5260 bp module with four genes: OXA-58, two IS30 family transposases, and a hypothetical protein. However, more work must be done to identify other *pdif* modules carrying genes not related to antibiotic resistance.

Which Plasmids Carry Antibiotic Resistance Genes?

As vehicles of horizontal gene transfer, plasmids play a crucial role in the dissemination of antibiotic resistance genes within pathogenic bacterial populations (San Millan, 2018; Carattoli, 2013). To evaluate the role of *A. baumannii* plasmids in the dispersion of antibiotic resistance genes, we searched for the presence of acquired resistance genes in our plasmid set using the ResFinder database (Zankari et al., 2012). In this manner, we identified not only plasmids that carry antibiotic resistance genes but also the plasmids lineages associated with these genes (**Supplementary Table S7**). Only 35.2% of our plasmid collection possesses antibiotic resistance genes, and of these plasmids, thirty-eight contain only one antibiotic resistance gene. Fifteen plasmids have two antibiotic resistance genes, and eight plasmids have three or more of these genes. The most frequent antibiotic resistance genes were those involved in resistance to aminoglycosides, which were present in 60.6% of the plasmids carrying antibiotic resistance, followed by plasmids with genes conferring resistance to beta-lactam antibiotics (49.1%). Sulfonamide resistance genes were also present in 26.2% of the plasmids with antibiotic resistance, and 14.7% have genes implicated in macrolide resistance.

Of the twenty-three plasmid lineages, only thirteen have members with antibiotic resistance genes. However, most commonly, only a few members of a plasmid lineage possess this type of gene, suggesting that these genes were secondary acquisitions after the origination of the lineage. With a few exceptions, antibiotic resistance genes are closely linked to one or two IS elements, in some cases to class 1 integrons, and in three plasmids, namely, pA85-3, pAB04-2 and pUSA15-1, all of which are members of LN_1, the antibiotic resistance genes are linked to an AbaR4 element (Hamidian et al., 2014; Hamidian and Hall, 2018b). A good example of this situation is lineage LN_1. This lineage has 42 members, but only 14 have antibiotic resistance genes, and of these plasmids, nine carry one antibiotic resistance gene; three plasmids have two resistance genes; and plasmid p1AB5075 carries eleven of these genes. One gene is an aminoglycoside resistance gene (*aph(3')-Via*) surrounded by two IS*Aba25* elements, and the remaining antibiotic resistance genes are class 1 integrons. The other twelve plasmids have antibiotic resistance genes tightly linked to IS*Aba1* or IS*Aba25* elements. These observations suggest that the IS elements and

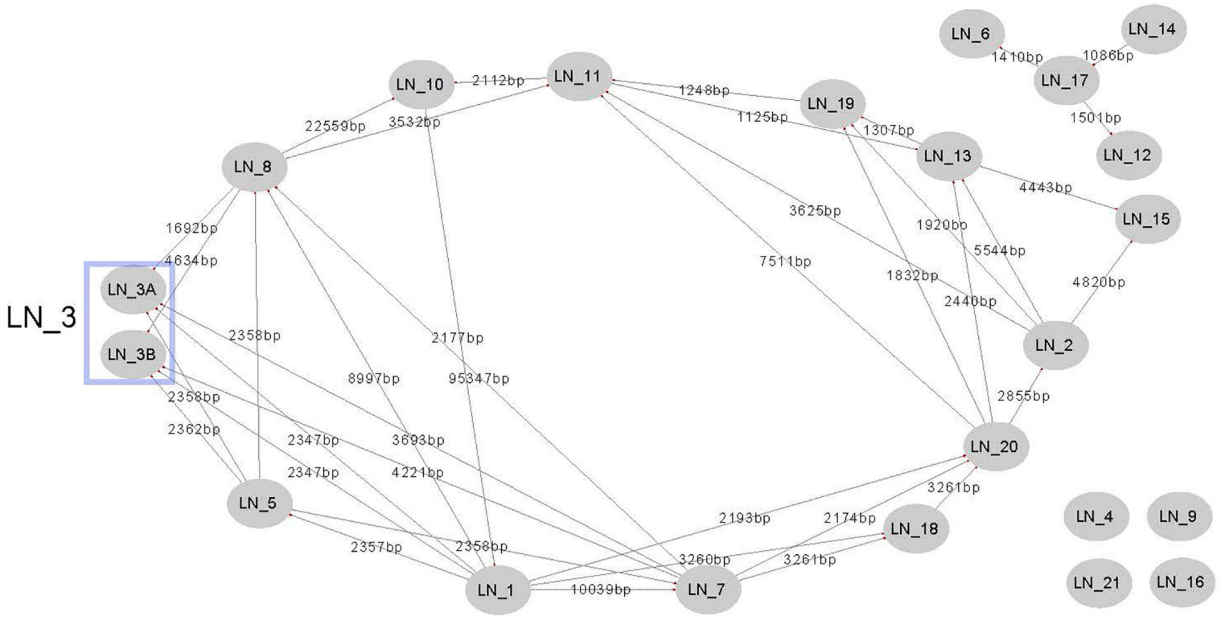


FIGURE 6 | Gene flux between plasmid lineages. Edges connect plasmid lineages that share at least 1Kb of DNA with an identity of 90%. Numbers in edges represent the total amount of different DNA sequences that members of one plasmid lineage have in common with members of the other plasmid lineage.

antibiotic resistance genes were acquired after the origin of this plasmid lineage.

The most predominant mechanism for carbapenem resistance in *A. baumannii* is the activity of OXA-type beta-lactamases (serine carbapenemases), some of which are encoded in plasmids (Da Silva and Domingues, 2016). In the analyzed plasmids, we found seven lineages with members carrying *bla*OXA genes: seven members of LN_1 carry *bla*OXA-23 genes as well as two members of LN_5. The four members of LN_6, all obtained from Mexican isolates, have *bla*OXA-72 genes. All members of LN_11 and LN21 possess *bla*OXA-58 genes, and one member of LN_14 and another from LN_17 contain *bla*OXA-24 genes.

Gene Flux Between Plasmid Lineages

To evaluate the gene flux between plasmid lineages or the amount of gene information that is shared between plasmid lineages, we performed BLASTn comparisons using the representative plasmid of one lineage as a query against all plasmids belonging to the other lineages. With this approach, we identified all DNA regions of 1 kb or higher with an identity of at least 90% and recorded the genes that remained in such regions. The results of this analysis are summarized in **Supplementary Table S8**. The amount of sequence information that two plasmid lineages can share varies dramatically (**Figure 6**). As described above, the lineage pair LN_7 and LN_8 share at least 90% sequence identity and coverage higher than 50% but lower than 85%. In contrast, lineages LN_4, LN_9, LN_16 and LN_21 do not share DNA sequences higher than 1 kb with any other plasmid lineage. Interestingly, plasmid members of these lineages are embedded in different genomic backgrounds, as illustrated in the phylogenetic tree shown in **Figure 7**. The remaining lineages share information with at least three and up to seven other plasmid lineages (**Supplementary Table S8**). Most of the DNA sequences that are shared between plasmid lineages, as expected, contain transposable elements, commonly but not exclusively IS*Aba1* and IS*Aba125*. Sets of antibiotic resistance genes are also frequently shared between plasmid lineages, and these genes are frequently linked to transposable elements such as IS elements and antibiotic resistance islands (AbaR4), suggesting that these elements frequently travel together (**Supplementary Table S6**).

Beyond *A. baumannii* and *Acinetobacter*

It has been shown that plasmids play a crucial role in disseminating virulence and antibiotic resistance genes in pathogenic bacteria. However, not all plasmids have the same potential to act as vectors for these purposes. One property that imposes limits on this potential is the replication host range. Some plasmids are capable of replicating in one or a few related species (narrow host range), while others are capable of replicating in an ample range of species and even genera (wide host range) (Jain and Srivastava, 2013). To evaluate the potential plasmid host ranges of the different *A. baumannii* plasmid lineages, we follow two strategies: first, we explored the NCBI nr (nonredundant) database by BLASTp analysis. We searched for proteins identical in sequence to those annotated as Rep proteins in our plasmid collection but excluded those identified in *A. baumannii* or *Acinetobacter*. Second, we also performed a BLASTn analysis of the NCBI nr (nonredundant) database, using the DNA sequences of the representative plasmids of each lineage and all orphan plasmids as queries but, again, excluding matches within *A. baumannii* or within the *Acinetobacter* genus.

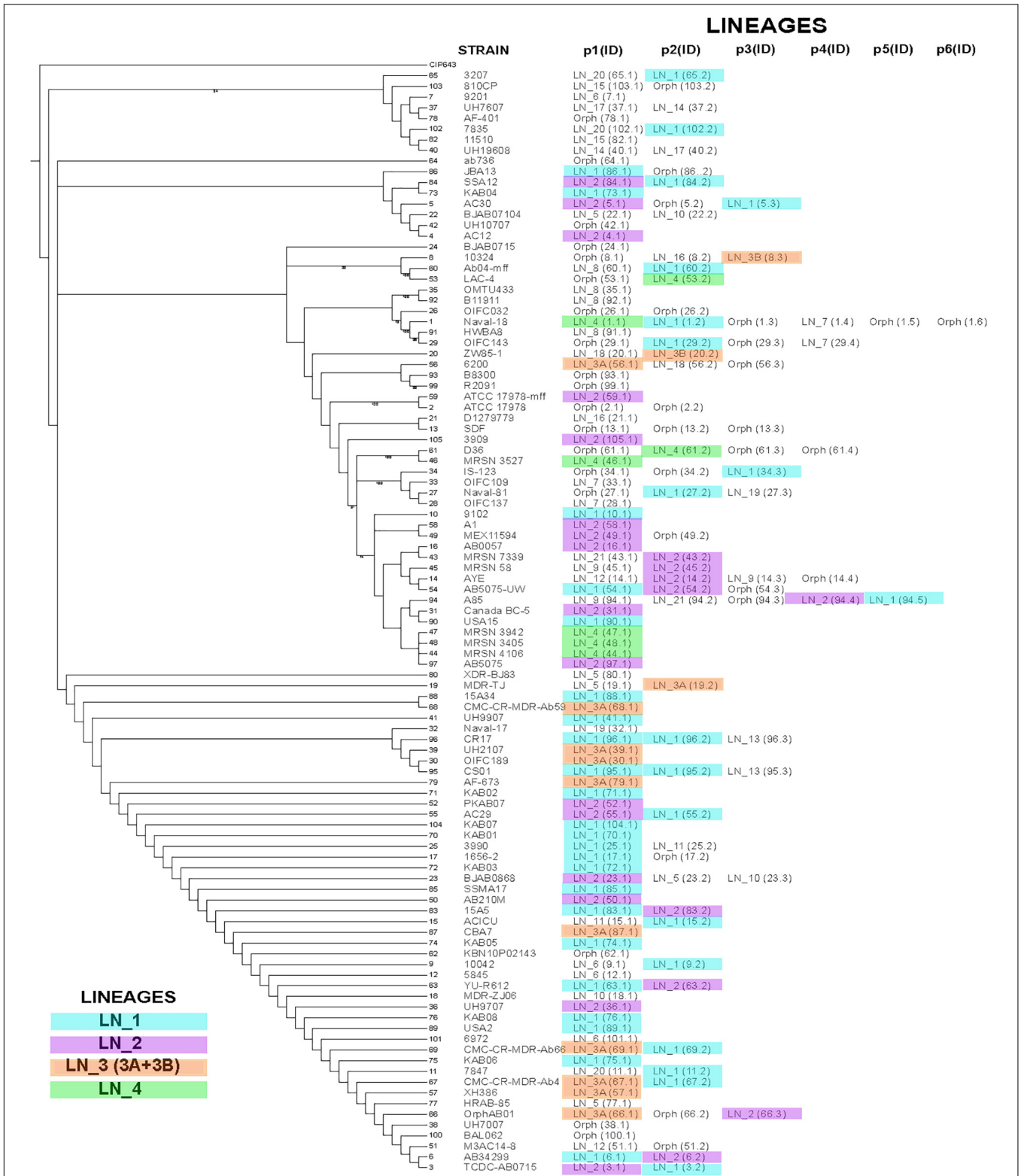


FIGURE 7 | Phylogenetic Tree of strains carrying the plasmids analyzed here. The tree was constructed using unicopy ribosomal protein genes without recombination signals. Bootstrap values higher than 70% are indicated in the tree. Strains containing our plasmid collection harbors a different numbers of plasmids, between one and six (p1..p6). At the right of each strain name shows the plasmids that that particular strain contains, using the alias plasmid name listed in **Supplementary Table S1** and the plasmid lineage that they belong. Plasmids marked in color belong to LN_1, LN_2, LN_3 or LN_4. Orph, indicates that the plasmid is an orphan.

A summary of our findings is presented in **Supplementary Table S9**. The Rep protein that seems to have a broad host range is encoded in the orphan plasmid pAB3 and can be found in the genomes of twelve genera of Gammaproteobacteria, ten genera of Betaproteobacteria, and three genera of Alphaproteobacteria and even in the actinobacterial species *Mycobacteroides abscessus*. This protein belongs to the RepC family. Some replication proteins of the GR3 homology group are also found in a wide variety of bacteria. For example, Rep proteins of the plasmids pB11911 (LN_8) and pMDR-ZJ06 (LN_10) were also identified in twelve different genera, all within Gammaproteobacteria. Similarly, the GR3 Rep protein of the orphan plasmid pHWBA8_1 was also found in the genomes of ten different genera of Gammaproteobacteria. Some Rep proteins of homology group GR2 were identified in, in addition to *A. baumannii, Enterococcus faecium, Klebsiella pneumoniae* and *Providencia rettgeri*. Some other Rep proteins were identified outside of Gammaproteobacteria; for example, some Rep proteins of LN_12 (GR11) and LN_14 (GR27) were located in *Neisseria meningitidis* (Betaproteobacteria). Additionally, the Rep protein of the orphan plasmid pIS123-12 (GR20) was present in the betaproteobacterial species *Nakamurella silvestris*. The remaining Rep proteins of the other GR groups seem to have a limited host range, being found in only *Acinetobacter*.

Intriguingly, some plasmids in our collection are very similar to other plasmids that are not closely related to *Acinetobacter*. For example, pMDR-ZJ06, which is the representative plasmid of LN_10, shares ≥ 75% coverage and 99% DNA sequence identity with the plasmid pEA49-KPC (GenBank: KU318419.1) of *Enterobacter aerogenes,* the plasmid RCS40_p (GenBank: LT985241.1) of *E. coli*, the plasmid pPMK1-NDM (GenBank: NZ_CP008933.1) of *K. pneumoniae* and the plasmid unnamed1 of *Citrobacter* sp. Similarly, plasmid pKP-NCGM38-1 (GenBank: AB825955.1) of *K. pneumoniae* shares 86% coverage and 99% identity with the representative plasmid of LN_13, indicating that this plasmid belongs to LN_13. The representative plasmid of LN_18 is almost identical to the plasmid p3SP-NDM (GenBank: KP900015.1) of *E. aerogenes* strain p3SP and shares 75% coverage and 99% identity with the plasmid p06-1619-NDM (GenBank: KX832928.1) of *P. rettgeri*.

The smallest plasmid in our collection, the orphan plasmid pJBA13_2 (1,109 bp), is almost identical to other very small plasmids belonging to other bacterial classes. This plasmid shares 100% coverage and 100% identity with an unnamed plasmid (GenBank: NZ_CP021055.1) from *Methylobacterium zatmanii* strain PSBB041 and with the plasmid unnamed6 (GenBank: NZ_CP023042.1) from *Komagataeibacter saccharivorans* CV1, both belonging to Alphaproteobacteria. Additionally, with plasmid unnamed2 (GenBank: NZ_CP013938.1) from *Weissella cibaria* strain CMU (Firmicutes) and with unnamed plasmid2 (GenBank: NZ_CP021993.1) from *Cryobacterium* sp. LW097 (Actinobacteria). Finally, orphan plasmid pJBA13_2 is almost identical to plasmids from *Salmonella enterica* subsp. *enterica* serovar Kentucky str. SA20030505 (GenBank: NZ_CP022501.1), *Pantoea ananatis* strain YJ76 (GenBank: NZ_CP022430.1) and *E. coli* strain HB-Coli0 (GenBank: NZ_CP020935.1) (Gammaproteobacteria). These observations indicate that the *A. baumannii* plasmid replication systems vary widely in host range; some seem to replicate only in *Acinetobacter* species, while others are capable of replicating in bacteria of different families and even different bacterial classes.

Pandemic and Epidemic Plasmids

We took two different approaches to evaluate whether our plasmid lineages are pandemic, that is, capable of existing in a wide range of chromosomal backgrounds, or epidemic, that is, only found in a few closely related chromosomes. For this purpose, we first determined the number of STs (Oxford and Pasteur MLST schemes) containing members of a specific plasmid lineage (listed in **Supplementary Table S1**). We found that a majority of our plasmid lineages occurred in more than one ST. Moreover, most of the plasmid lineages are present not only in isolates belonging to the International Clones but also out of these clonal complexes. For example, members of LN_1 are present in 20 different STs, and members of LN_2 are present in 9 STs. These lineages are clearly pandemic; however, members of some plasmid lineages seem to be epidemic, considering that these plasmids are restricted to a few STs; for instance, LN_3A, possessing 11 members, is represented in only 3 STs, mostly in ST208, and members of LN_4 are located in 3 STs. Lineages LN_9, LN_11, LN_14, LN_15, and LN_17 are present in one ST, but these lineages have only two or three members each, and in these circumstances, it is not possible to determine whether these lineages have a restricted chromosomal range.

Our second approach was to construct a phylogenetic tree using single-copy ribosomal genes without recombination signals of the strains including our plasmid collection and map the different plasmid lineages in this tree. In **Figure 5** we show the locations in the tree of our entire plasmid set, indicating the corresponding plasmid lineages. In **Figure 5**, we show evidence that the members of the four largest lineages (LN_1 to LN_4) are scattered throughout the phylogenetic tree, indicating that these plasmids are capable of replicating in a wide range of chromosomal backgrounds that are not necessarily closely related. However, notably, despite the wide distribution of plasmids belonging to LN_2 and LN_3A, these plasmids do not possess genes annotated as part of the conjugation or mobilization machineries. Nevertheless, as mentioned above, all members of LN_2 have *oriT*-like sequences that probably can be used for mobilization when they co-reside with a compatible conjugative helper plasmid.

Our bioinformatics analyses suggest that *A. baumannii* plasmids have diverse host ranges: plasmid lineages containing a Rep protein of homology group GR3, have the potential to replicate in an extensive range of bacterial genera, including some important pathogens such as *K. pneumoniae, E. coli,* and *Salmonella enterica.* As described above, the representative plasmid of LN_10 is very similar in sequence and gene content to previously described plasmids of *E. aerogenes, E. coli* and *K. pneumoniae.* All these plasmids of presumably of very wide host ranges are located in not closely related clades in the phylogenetic tree, suggesting that these plasmids were introduced into the *A. baumannii* populations in different independent

events. The remaining plasmids seem to replicate only within *Acinetobacter* (restricted host range).

A notable feature that we want to point out is the behavior of plasmids as antibiotic resistance gene carriers: members of lineages LN_4, LN_6, LN_7, LN_8, LN_10, LN_11, LN_3B, LN_18 and LN_20 all carry antibiotic resistance genes. As mentioned above, lineages LN_8 and LN_10 can probably also replicate in *K. pneumoniae*, a pathogen that has been identified as an important reservoir of antibiotic resistance genes (Wyres and Holt, 2018). In contrast, lineages LN_2, LN_3A LN_12, LN_13, LN_15, LN_16A, LN_19, and LN_21 do not carry genes of this type. We also found plasmids with intermediate behavior, in which some members of the lineage carry antibiotic resistance genes, while others do not (LN_1, LN_5, LN_14 and LN_17). At least in LN_1 and LN_5, antibiotic resistant genes are closely linked with IS elements.

Evolution of *A. baumannii* Plasmids in the Nosocomial Environment

Considering all these observations as a whole, we want to propose the following hypothesis to explain the evolution of *A. baumannii* plasmids in the nosocomial environment: before the advent of antibiotics, *A. baumannii* plasmids were parasites of this organism. The gene of these plasmids were involved not only in maintenance functions but also in reducing the fitness cost of plasmid replication. The stability of the structure and gene content of these plasmids over long periods of time in several genetic backgrounds within each of plasmid lineage is probably a product of this condition. When *A. baumannii* arrived in the nosocomial environment, this species began to interact with other bacterial pathogens, such as *K. pneumoniae* or *E. coli,* which already contained plasmids with antibiotic resistance genes. At this point, *A. baumannii* acquired a subset of these plasmids with broad host ranges, probably containing Rep proteins of the homology group GR3. The coexistence of these broad-host-range plasmids with the *A. baumannii* genome allowed the dispersion of new transposable elements with or without antibiotic resistance genes. The acquisition of IS elements permitted some plasticity in *A. baumannii* plasmids. In other words, we propose that at the beginning, *A. baumannii* plasmids were specialized to replicate in this microorganism with a minimal fitness cost, but the acquisition of new broad-host-range plasmids that already contained antibiotic resistance genes native to other microbial pathogens allowed *A. baumannii* to survive easily in the nosocomial environment and become a pathogen of concern.

A Note Regarding Plasmid Nomenclature

During this study, we found that the nomenclature of *Acinetobacter* plasmids does not follow any type of rule. Moreover, adding an additional layer of complexity, some plasmids do not have official names and are simple referred to in GenBank as unnamed plasmids or tagged as p1, p2, etc. This evident lack of convention imposes unnecessary challenges during a systematic study of plasmids. We need names that easily link a plasmid with its strain/isolate ID and with the species name. For these reasons, we strongly suggest naming *Acinetobacter* plasmids by following the nomenclature rules proposed for the *Agrobacterium* and *Rhizobium* cryptic plasmids: first, all plasmid names must begin with letter "p" followed by the first letter of the genus name and the first two letters of the species name. Then, the strain/isolate ID number is added, followed by a lower-case letter, using "a" for the smallest plasmid, "b" for the next plasmid and so on. For example, the name of the smallest plasmid of *A. haemolyticus* MC1956 would be pAhaMC1956a. The plasmid that is next in size in the same strain will be pAhaMC1956b, and so on.

The annotation of plasmid genes is also confusing and not uniform, and genes are often annotated by using the name of the best BLAST hit and not the true biological function of the gene in the plasmid. Recently, Christopher M. Thomas and coworkers published a paper addressing all these problems and suggested methods to resolve these issues. We encourage scientists interested in plasmid biology to follow those recommendations (Thomas et al., 2017).

CONCLUSION

Acinetobacter baumannii plasmids belong to a limited number of plasmid lineages and their structure seem to be very stable, in contrast to the observations made in the so-called mosaic plasmids. Mosaic plasmids are composed of genetic elements from distinct sources and they are highly dynamic in acquisition and loss of genes (Pesesky et al., 2019).

Core genomes of *A. baumannii* plasmid lineages contain more genes to those required for plasmid maintenance functions and these genes seems to be not related to the nosocomial environment, open the possibility that they could have other functions and opening the possibility that they reduce fitness cost in the plasmid host. Evidence showed here, suggest that each plasmid lineage represents a plasmid incompatibility group and that the largest plasmid lineages are widely distributed along the phylogenetic tree even though, some of them lack identifiable mobilization systems. In most plasmid lineages transposable elements and antibiotic resistance genes are secondary acquisitions. Plasmids of broad host range have a crucial role in the acquisition of antibiotic resistance genes in *A. baumannii.*

MATERIALS AND METHODS

Plasmid Collection

Our collection included all the complete plasmids (with the "assembled molecule" status) of *A. baumannii* available in the RefSeq and GenBank databases (NCBI) on August 14th, 2017. We parsed the GenBank and fasta files with the SeqIO Biopython module (Cock et al., 2009) in Python 2.7 for all subsequent analyses.

To increase the diversity of our plasmid collection, we obtained the complete genome sequences of 10 Mexican isolates using the PacBio RSII and Illumina NextSeq platforms.

The genome sequences of three isolates, namely, 7804, 810CP and 3207, have previously been reported by some of the authors of this manuscript (Castro-Jaimes et al., 2016; Pérez-Oseguera et al., 2017).

For the other eight isolates, we constructed hybrid assemblies with reads from both platforms using SPAdes v3.9.0 or Unicycler v0.4.1 (Bankevich et al., 2012; Wick et al., 2017). We performed functional annotation with the NCBI Prokaryotic Genome Annotation Pipeline. The GenBank accession numbers of the genomes of the Mexican isolates are listed in **Supplementary Table S10**. Therefore, in total, we analyzed 173 complete plasmids, and the complete list of plasmids and strains is shown in **Supplementary Table S1**.

Plasmid Lineage Delimitation

We performed paired BLASTn (Camacho et al., 2009) searches between all 173 complete plasmids in our collection. We built different plasmid networks, each based on a defined range of coverage (from 40 to 90%). For each plasmid pair, we placed a link between the plasmids if the smallest plasmid covered at least a defined percentage of the other plasmid, where coverage was determined by the sum of alignment lengths with greater than 90% identity Then, we extracted the islands or "connected components" with NetworkX (Hagberg et al., 2008) in Python 2.7. For each connected component, we extracted the most connected plasmid (hub) to use as a reference. When there was more than one hub, we sorted the hubs by size and selected the largest plasmid. The plasmid lineages and the associated references are listed in **Supplementary Table S1**.

Extraction of Plasmid Replication Proteins

We used an annotation-based approach to extract the plasmid initiation replication proteins. By using the plasmid GenBank files, we performed a case-insensitive search for the following keywords in the products: "replication protein", "plasmid replication initiator", "plasmid replication", "DNA replication", "plasmid replicase", "replication a", "replication b", "replication c", "RepB", "rolling circle", "replication initiation", "replicase". Then, we extracted both the nucleotide and protein sequences and excluded partial genes and pseudogenes. Additionally, we extracted 500 nucleotides upstream and downstream of the Rep gene for further analyses. This entire process was performed with Python 2.7 and the Biopython SeqIO module (Cock et al., 2009).

Reference Proteins for Homology Group Designation

We compiled all replication (Rep) proteins that were reported by Bertini et al. (2010); (Bertini et al., 2010) by gene name, plasmid name and plasmid accession number when available. For those cases in which the Rep proteins did not have a locus tag or gene name, we added an artificial locus tag built using the replicon ID and the replicase name. When the replicase name was not available, we assigned the word 'rep' followed by a number in the order of appearance in the GenBank file to distinguish between replicases. In some cases, when there were two replication proteins in the same plasmid, to correctly assign these proteins as references for certain homology groups, we performed a BLAST search of these proteins against the GenBank nr database to identify corresponding hits outside the *Acinetobacter* genus reported in **Supplementary Table S1** in Bertini et al. (2010). Two proteins could not be identified: the Aci3 replicase from plasmid Ab599 (member of GR3), because the plasmid sequence was not deposited in databases, and the Aci2 replicase from the MAD plasmid, because the plasmid had a partial sequence that did not include the replicase. Therefore, we omitted these proteins from our analyses and examined other members of the same homology groups instead. As reported by Lean and Yeo (2017), the GR2 homology group should be split into two groups; therefore, we separated the proteins that represent GR2 from those of the newly formed GR20. Additionally, (Cameranesi et al., 2017) recently reported new homology groups; thus, we downloaded the plasmids that harbored the replicases that represent these groups and extracted those genes. **Supplementary Table S2** lists all proteins used as references in this work, including the origins, accessions, numbers and headers used in the multi-FASTA files included in **Supplementary Materials S1**, **S2**.

Homology Group Assignation for Rep Proteins

First, we performed paired BLASTn (Camacho et al., 2009) searches between all genes encoding replication initiation proteins present in our plasmid collection. We retained hits with more than 74% nucleotide identity and that covered at least 90% of the query. Then, if the query coding sequence (CDS) mapped to only one homology group, we designated the sequence as belonging to that group, whereas if there was more than one hit for different homology groups, we assigned the query to the GR with the highest percentage identity. We discarded the GR23 homology group because the associated reference (KY984047_repAci23) was 100% identical to one of the references of GR8 (GU979000.1_p11921_repA).

Plasmid Rep Protein Phylogenetic Analysis and Designation of New Homology Groups

We built a network in which each gene encoding a Rep protein was connected to another if the two genes shared at least 74% nucleotide identity and 90% coverage. Then, for the islands or connected components that did not have a Rep protein in the reference table, we selected the hub as a reference and added it to **Supplementary Table S1**. Additionally, we built plasmid replication initiation protein phylogenies to validate current assignations and new homology groups. We searched for the associated Pfam domains in the Pfam database (El-Gebali et al., 2019), accessed on February 21st, 2018, to separate the proteins by conserved domains and perform alignments separately because these proteins are very different. We used Clustal Omega (Sievers et al., 2014) to align amino acids and RevTrans (Wernersson and Pedersen, 2003) to guide the nucleotide alignment by the translated CDS. Then, we ran jModelTest2 (Darriba et al., 2012) to search for an adequate evolutionary model and built the

phylogenetic tree with PHYML (Guindon and Gascuel, 2003) with the selected model. By visual inspection, we validated the references of new homology groups, selected proteins that may be representative of new clades and designated these proteins as new homology groups, as detailed in **Supplementary Table S2**.

Phylogenetic Analysis of Ribosomal Proteins and MLST

We used Roary (Page et al., 2015) to extract monocopy genes encoding ribosomal proteins belonging to the core genome and that had the exact same size in all the strains to avoid gaps in the alignment. We aligned the ribosomal proteins with Clustal Omega (Sievers et al., 2014) to guide the nucleotide alignment with RevTrans (Wernersson and Pedersen, 2003). We discarded sequences with recombination signals detected with RDP4 (Martin et al., 2015). We concatenated the remaining nucleotide alignments with FASconCAT-G (Kück and Longo, 2014) and used jModelTest2 (Darriba et al., 2012) to select the evolutionary model to build a phylogenetic tree with PHYML (Guindon and Gascuel, 2003). We used the ribosomal proteins of the *Acinetobacter haemolyticus* CIP 64.3 strain as an outgroup. The sequence type (ST) assignation of each *A. baumannii* isolate, under Oxford and Pasteur MLST schemes, were obtained from the PubMLST database[1] (Bartual et al., 2005; Diancourt et al., 2010).

Identification of Secretion Systems, Antibiotic Resistance Genes, and Insertion Sequences on Plasmids

We used MacSyFinder with the TXSSCAN profiles (Abby and Rocha, 2017) to identify secretion systems on the plasmid collection and ResFinder to identify the acquired antibiotic resistance genes present in our plasmid set (Zankari et al., 2012). We identified the insertion sequence (IS) elements present in the plasmids using the ISfinder database at[2] (Siguier et al., 2006).

Identification of *pdif* Sites (XerC/D and Xer D/C) on Plasmids

To identify the *pdif* sites in our plasmid set, we made a BLASTn analysis using as queries the *pdif* sites of plasmid pS30-1: XerC/D, ATTTCGTATAAGGTGTATTAT- GTTAATT and XerD/C, ATTTAACATAATGGCTGTTATGCGAAAC (Blackwell and Hall, 2017).

COG Assignments

We determined homologous gene assignments for each plasmid based on hidden Markov model (HMM) searches using the *hmmsearch* program (Eddy, 2011). This HMM search process employs a previously constructed model set that represents each of the 4873 COGs and 8539 Remained Orthologous Groups (ROGs) (Tatusov et al., 2003; Taboada et al., 2010). Then, using Perl scripts, we classified each assigned COG by using the general classification scheme of Tatusov [66]. We calculated the frequency of each gene per class and plotted the results using ggplot2 R scripts[3] (Wickham, 2009).

[1]https://pubmlst.org

[2]http://www-is.biotoul.fr

DATA AVAILABILITY STATEMENT

Genome sequences were deposited in NCBI/GenBank with the following accession numbers: Isolate 7847: NZ_CP023031.1, CP023032.1, CP023033.1. Isolate 7835: CP033243.1, CP03 3244.1, CP033245.1. Isolate 9102: CP023029.1, CP023030.1. Isolate 5845: NZ_CP023034.1, CP023035.1. Isolate 10042: NZ_CP023026.1, CP023027.1, CP023028.1. Isolate 10324: NZ_CP023022.1, CP023023.1, CP023024.1, CP023025.1. Isolate 9201: NZ_CP023020.1, CP023021.1.

AUTHOR CONTRIBUTIONS

MC conceived, designed, and coordinated the study. ÁP-O made plasmid profile analysis and genome analysis of Mexican isolates. AS-C and SC-J made genome assemblies, genome annotation, network analysis, and bioinformatics analysis, and made bioinformatic analysis. AS-C designed figures and most of the tables of the manuscript. R-MG-R made COG analysis and statistics. LA-P made the analysis of Rep proteins. LL made bioinformatic analysis and made many pf Perl scripts used in this work. PV contributed with the Mexican isolates, participated in the manuscript drafting and in the general discussion. SC-R and JS-S had a crucial role in the general discussion. All authors contributed to manuscript revision, read and approved the submitted version.

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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.2020.01283/full#supplementary-material

[3]http://www.R-project.org/

FIGURE S1 | Plasmid networks.

FIGURE S2 | Circular maps of members of plasmid lineage LN_3.

FIGURE S3 | Circular maps of members of plasmid lineages LN_4 and LN_5.

FIGURE S4 | Circular maps of members of plasmid lineages LN_6 and LN_7.

FIGURE S5 | Circular maps of members of plasmid lineage LN_8.

FIGURE S6 | Circular maps of members of plasmid lineages LN_9, LN_10.

FIGURE S7 | Maps of members of plasmid LN_11 and LN_12.

FIGURE S8 | Linear maps of members of plasmid lineages LN_13, and LN_14.

FIGURE S9 | Linear maps of members of plasmid lineages LN_15, and LN_16.

Figure S10 | Maps of members of plasmid lineages LN_17 and LN_18.

FIGURE S11 | Linear maps of members of plasmid lineages LN_19, and LN_20.

FIGURE S12 | Linear maps of members of plasmid lineage LN_21.

FIGURE S13 | Phylogenetic tree of Rep proteins belonging to the Pri_CT family.

FIGURE S14 | Phylogenetic Tree of strains carrying the plasmids analyzed here and their associated Toxin-Antitoxin modules.

TABLE S1 | List of plasmids belonging to lineages and their general characteristics.

TABLE S2 | Replicases used to assign GR homology groups.

TABLE S3 | Replicon-associated iterons identified in new GR members.

TABLE S4 | Plasmids encoding two or more replication proteins.

TABLE S5 | Conjugation genes and Toxin-Antitoxin system present in plasmids.

TABLE S6 | IS elements present in plasmids.

TABLE S7 | Antibiotic resistance genes located in plasmids.

TABLE S8 | Gene flux between plasmid lineages.

TABLE S9 | Replication proteins of A. baumannii plasmids which are identical in sequence to replication proteins in the genomes of other Acinetobacterspecies and in other genera.

TABLE S10 | GenBank accession numbers of Mexican isolates and their Sequence type following Oxfordand Pasteur schemes.

MATERIAL S1 | List in fasta format of genes encoding representative replication proteins of each one of the GR homology groups.

MATERIAL S2 | List in fasta format of the representative replication proteins of each one of the GR homology groups.

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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.

Apéndice 8 - High mortality in an outbreak of multidrug resistant *Acinetobacter baumannii* infection introduced to an oncological hospital by a patient transferred from a general hospital.

Cornejo-Juárez, P., Cevallos, M. A., Castro-Jaimes, S., Castillo-Ramírez, S., Velázquez-Acosta, C., Martínez-Oliva, D., et al. (2020). High mortality in an outbreak of multidrug resistant *Acinetobacter baumannii* infection introduced to an oncological hospital by a patient transferred from a general hospital. *PLoS One* 15, e0234684. doi:10.1371/journal.pone.0234684.

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RESEARCH ARTICLE

High mortality in an outbreak of multidrug resistant *Acinetobacter baumannii* infection introduced to an oncological hospital by a patient transferred from a general hospital

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Abstract

Objective

To describe the clinical features, outcomes, and molecular epidemiology of an outbreak of multidrug resistant (MDR) *A. baumannii*.

Methods

We performed a retrospective analysis of all MDR *A. baumannii* isolates recovered during an outbreak from 2011 to 2015 in a tertiary care cancer hospital. Cases were classified as colonized or infected. We determined sequence types following the Bartual scheme and plasmid profiles.

Results

There were 106 strains of *A. baumannii* isolated during the study period. Sixty-six (62.3%) were considered as infection and 40 (37.7%) as colonization. The index case, identified by molecular epidemiology, was a patient with a drain transferred from a hospital outside Mexico City. Ninety-eight additional cases had the same MultiLocus Sequence Typing (MLST) 758, of which 94 also had the same plasmid profile, two had an extra plasmid, and two had a different plasmid. The remaining seven isolates belonged to different MLSTs. Fifty-three patients (50%) died within 30 days of *A. baumanniii* isolation: 28 (20%) in colonized and 45 (68.2%) in those classified as infection ($p<0.001$). In multivariate regression analysis, clinical infection and patients with hematologic neoplasm, predicted 30-day mortality. The molecular epidemiology of this outbreak showed the threat posed by the introduction of MDR strains from other institutions in a hospital of immunosuppressed patients and

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highlights the importance of adhering to preventive measures, including contact isolation, when admitting patients with draining wounds who have been hospitalized in other institutions.

Introduction

Acinetobacter baumannii has become a major hospital pathogen, due to multidrug resistant (MDR) strains and it is now considered one of the six most important microorganisms that causes hospital-acquired infections worldwide, with attributable mortality ranging from 8% to 35% according to strain and type of infection as well as increase in hospital stays and health care expenditures [1,2]. Bloodstream infection (BSI) and pneumonia, especially ventilator-associated pneumonia (VAP), are the most severe infections caused by this gram-negative bacterium [3,4].

Carbapenems have been considered the drugs of choice to treat *A*. *baumannii* infections. These drugs are still the first-line agents for empirical therapy in areas with high rates of susceptibility [3]. However, the extensive use of carbapenems for the treatment of cephalosporin-resistant *Enterobacteriaceae* has been regarded as one of the main risk factors promoting the emergence and spread of multidrug-resistant (MDR) *A*. *baumannii* strains.

The role of the environmental contamination in the transmission of nosocomial infections in general and in *A*. *baumannii* infections in particular is well recognized [5]. This pathogen is able to grow at various temperatures and pH conditions, so it has the ability to persist in either moist or dry conditions in the hospital environment, thereby contributing to transmission [6, 7, 8]. We documented an outbreak of an MDR *A*. *baumannii* strain identified initially in 2011 in the intensive care unit (ICU) that lasted until 2015. The strain was introduced by an infected patient transferred from another hospital. This study aimed to identify the epidemiology of the outbreak and to describe the clinical evolution and risk factors for adverse outcomes of patients during the outbreak of MDR *A*. *baumannii*.

Methods

We conducted a retrospective study at Instituto Nacional de Cancerología (INCan), a 135-bed tertiary care oncology hospital located in Mexico City, with an average of 170,000 medical visits and 7,500 hospital discharges per year, taking care mainly of uninsured patients, and patients covered by the "popular insurance" design to offer a basic package of services for certain malignancies. The ICU has six beds, with a mean of 230 hospitalizations per year; 58% of them are surgical patients with solid tumors, 25% have hematologic malignancies, and 17% are patients with solid tumors on chemotherapy.

In March 2011, we isolated the first strain of MDR *A*. *baumannii*; since then, multiple isolates from different sources of hospitalized patients at the ICU were recovered. No MDR *A*. *baumanii* strains had been isolated previously in the hospital. During 2011 to 2012, 78 isolates were identified (73.6%), decreasing the number of isolates recovered in the coming three years, until 2015. From January 2011 to December 2015, 106 patients with MDR *A*. *baumannii* isolates were identified, all were included in the study. Each isolate was classified as infected or colonized according to the criteria of two independent infectious diseases (ID) clinicians. A case was defined when the patient exhibited signs and symptoms of infection with or without a systemic inflammatory response. Bloodstream infection (BSI) was defined as laboratory-confirmed isolation of *A*. *baumannii* from blood cultures. Pneumonia was considered when new

or progressive infiltrates on x-ray appeared with at least two of the following parameters: fever (>38˚C) or hypothermia (<35.5˚C), leukocytosis (>12,000 cells/ml) or leukopenia (<4000 cells/ml), and positive bronchial aspirate culture [9]. Urinary tract infection was considered in those patients who present dysuria or hematuria and frequency or urgency, and urinalysis with pyuria and bacteriuria or positive nitrites. Surgical site infection defined as the infection in the part of the body where the surgery took place and occurring within 30 days after the operation. Airway colonization was defined if *A*. *baumannii* was isolated without symptoms of infection, and did not receive specific antimicrobial therapy. If there was a disagreement between the two physicians, a third ID physician analyzed the case, and a consensus was reached.

Clinical information was collected from the medical charts, including type of oncological disease, clinical stage (recent diagnosis, progression, relapse, partial or complete remission), recent chemotherapy or surgery (less than one month), recent hospitalization or antimicrobial use (previous 3 months), length of hospital stay, ICU admission and length of ICU stay, SOFA (Sequential Organ Failure Assessment) at ICU admission and at the date from *A*. *baumannii* culture was taken, days on mechanical ventilation, antimicrobial regimen, and outcome at 72 hours and at 30 days (alive, death attributable to infection, death related to neoplasm, or death due to other cause). Appropriate antimicrobial regimen was considered if it included colistin or tigecycline for ≥48 hours within the first 24 hours from isolation.

A.*baumannii* was isolated from clinical specimens submitted to the microbiology laboratory; identification and antimicrobial susceptibility were determined using the Gram-Negative Identification Panel (BDPhoenix™ automated system, New Jersey, USA). Identification of genotypic strains was confirmed by matrix-assisted laser desorption and ionization-time of flight mass spectrometry (MALDI-TOF-MS; Microflex, USA). MDR strains were tested for antibiotic disks on Mueller-Hinton agar containing colony suspension of *A*. *baumannii* equivalent to a 0.5 McFarland, Clinical and Laboratory Standards Institute (CLSI) recommendations for susceptibility [10]. Antibiotic disks included ceftriaxone (30 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), imipenem (10 μg), meropenem (10 μg), gentamicin (10 μg), and sulfamethoxazole/trimethoprim (SXT-1.25/23.75 μg) (CLSI). Colistin (10 μg) disks were tested although no parameters had been determined by CLSI. All isolates had an inhibition zone diameter ≥21 mm. [10]. Strains are considered MDR if they showed resistance to more than one agent in ≥3 antimicrobial categories such as aminoglycosides, antipseudomonal carbapenems and/or cephalosporins, fluoroquinolones, beta-lactam-beta-lactamase inhibitors, folate pathway inhibitors, and tetracyclines (Magiorakos criteria) [11]. All the strains included in this study, were resistant to all previous antibiotics families except tetracyclines and colistin. MICs reported were: ceftriaxone >32, ceftazidime >2, ciprofloxacin >2, imipenem >8, meropenem >8, gentamicin >8, and SXT >2/38. Strains were preserved and stored in glycerol at -70˚C for further analysis.

DNA was extracted from a representative strain of each pattern (Genomic DNA Purification Kit, Thermo Scientific) for subsequent PCRs. First, rpoB's Zone-1 [12] was amplified and sequenced to confirm taxonomic status. Plasmid profiles of each isolate were obtained during the study period with the modified Eckhardt protocol (S1 File) [13,14]. Each plasmid pattern was given an arbitrary roman numeral and used to group the strains. Strains confirmed as *A*. *baumannii* by their best hit against the NCBI database (at least 99% identity) were subject to MultiLocus Sequence Typing (MLST) scheme (Bartual) [15]. All PCR fragments were cloned in pJET1.2 (Thermo Scientific) and subject to Sanger sequencing with pJET universal oligonucleotides. *A*. *baumannii* MLST sequences were queried against the pubMLST database (http://pubmlst.org) to get their sequence type (ST). An ST number was requested to pubMLST for each one of the isolates with a new MLST profile.

Statistical analysis

Categorical variables were described by frequencies and percentages. Quantitative variables were described by means, standard deviation, medians, and quartiles according to distribution. Univariate analysis was performed using Student's *t*-test or Mann-Whitney U test for continuous variables, and Chi-square or Fisher's exact test was used to compare categorical variables. Variables with P values $\leq$0.5 in the univariate analysis were included in the multivariate analysis. Rates of overall survival were estimated using the Kaplan Meier method and log rank test. P values $<$0.05 were considered statistically significant. Data were analyzed using STATA (ver. 14) software.

Ethics

The study was approved by the INCan Ethics Review Board (Rev/0009/19). No consent form was required; patient´s information was anonymous and de-identified prior to analysis.

Results

During the study period, 108 patients with MDR *A*. *baumannii* isolates were identified: two were excluded because *rpoB* sequencing one was identified as *A*. *haemolyticus* and one as *Acinetobacter spp*. There were included 106 patients with isolates from the following sources: blood (n = 18), bronchial aspirates (n = 49), surgical site infection (n = 20), urine (n = 12), pleural fluid (n = 4), biopsies (n = 2) and catheter tip (n = 1). Fourteen patients had MDR-*A*. *baumannii* isolated from two different clinical sites and three patients had the isolation from three different sites. Forty-one strains were isolated in 2011 (37.7%), 37 in 2012 (34.9%), 12 in 2013 (11.3%), 13 in 2014 (12.2%), and 3 in 2015 (2.83%). Sixty-six (62.3%) isolates were considered as infection and 40 (37.7%) were classified as colonization (Fig 1).

The index case was identified by molecular epidemiological analysis; was an infected hemato-oncological patient with a pleural drainage, who was transferred from another hospital outside Mexico City.

Of all the isolates obtained during the outbreak, 99 had the same sequence type MLST 758; 94 had the same plasmid profile as the one recovered from the index case, two had the same plasmid an extra plasmid, and two others were also MLST 758 but had different plasmid profiles. Seven had different MLST (four of them proceeded from infected patients and had been hospitalized in another hospital within the previous 60 days).

Representative plasma profiles of selected *Acinetobacter* strains are shown in Fig 2.

From the whole group, there were 59 men (55.6%); the mean age was 48.3 ± 16 years. Half of patients had a hematologic malignancy (n = 52, 49.1%), 66 (62.3%) had recent cancer diagnosis, 44 (41.5%) were receiving chemotherapy, and 28 (26.4%) had major surgery. Hematologic malignancies were more common in the infected group (76.9%) than in the colonization group (30%, p = 0.002). Also, there were more patients with hematologic malignancies classified as infected (60.6%) compared with patients with solid tumors (39.4%, p = 0.002). In the infected group, there were more patients (28.8%) who had received broad-spectrum antimicrobials during the previous three months vs. the colonization group (17.5%, p = 0.048). Other clinical and demographic characteristics are shown in Table 1.

Sixty-six patients (62.2%) were hospitalized in the ICU; the most common causes of ICU admission were: septic shock in 25 patients (37.9%) (eight related to *A*. *baumannii* infection, median of 12 days of hospitalization), and respiratory failure in 20 patients (30.3%). SOFA score at admission was not different between infected vs. colonized patients (p = 0.344); however, it was significantly higher in infected vs. colonized patients at the day in which the sample with *A*. *baumannii* isolation was taken (median 7 vs. 0, p $<$ 0.001). Sixty-six patients (62.3%)

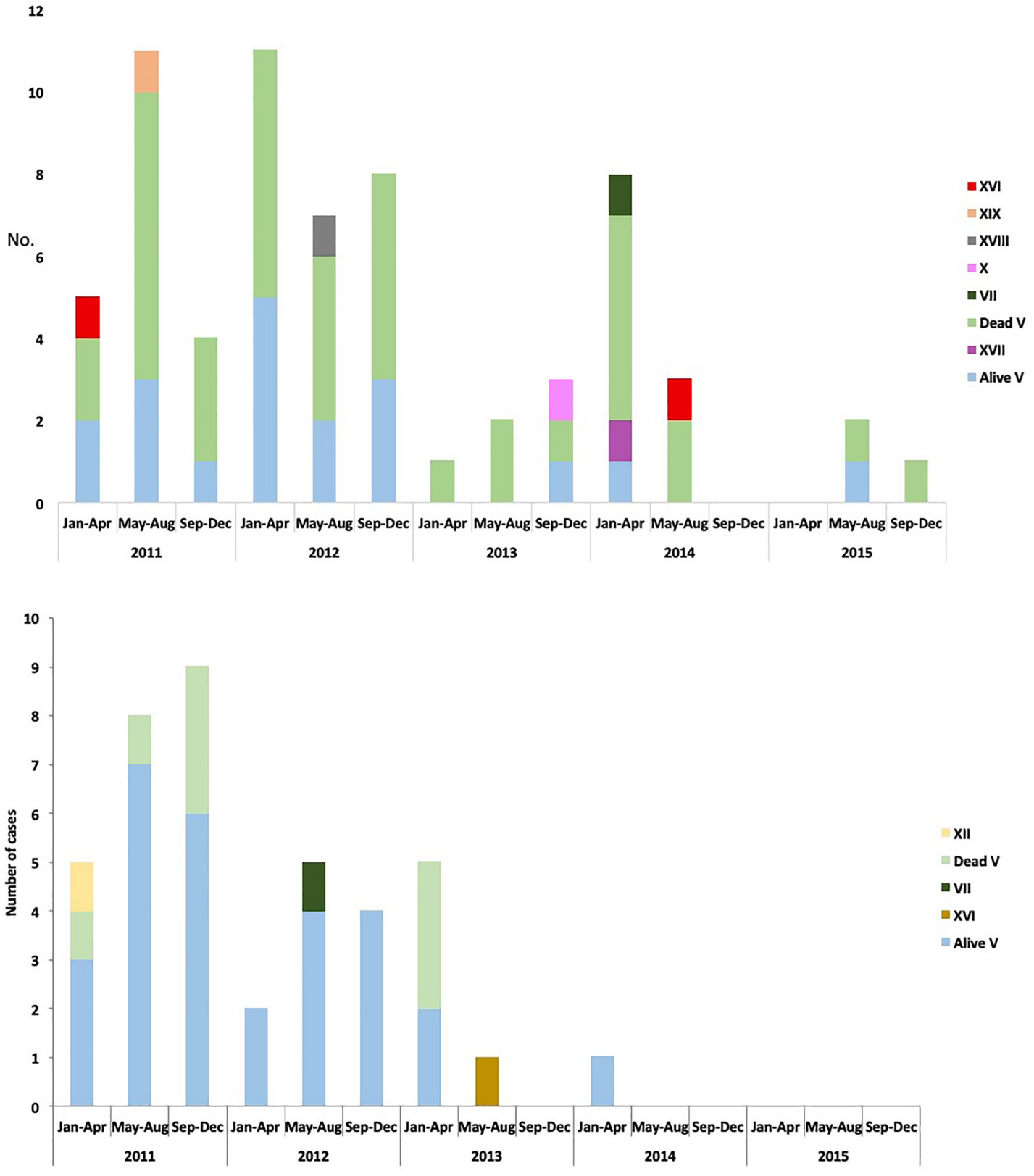

Fig 1. Number of infected (1a) and colonized (1b) patients during the study period (2011–2015) divided into alive or dead, including the plasmid profile.

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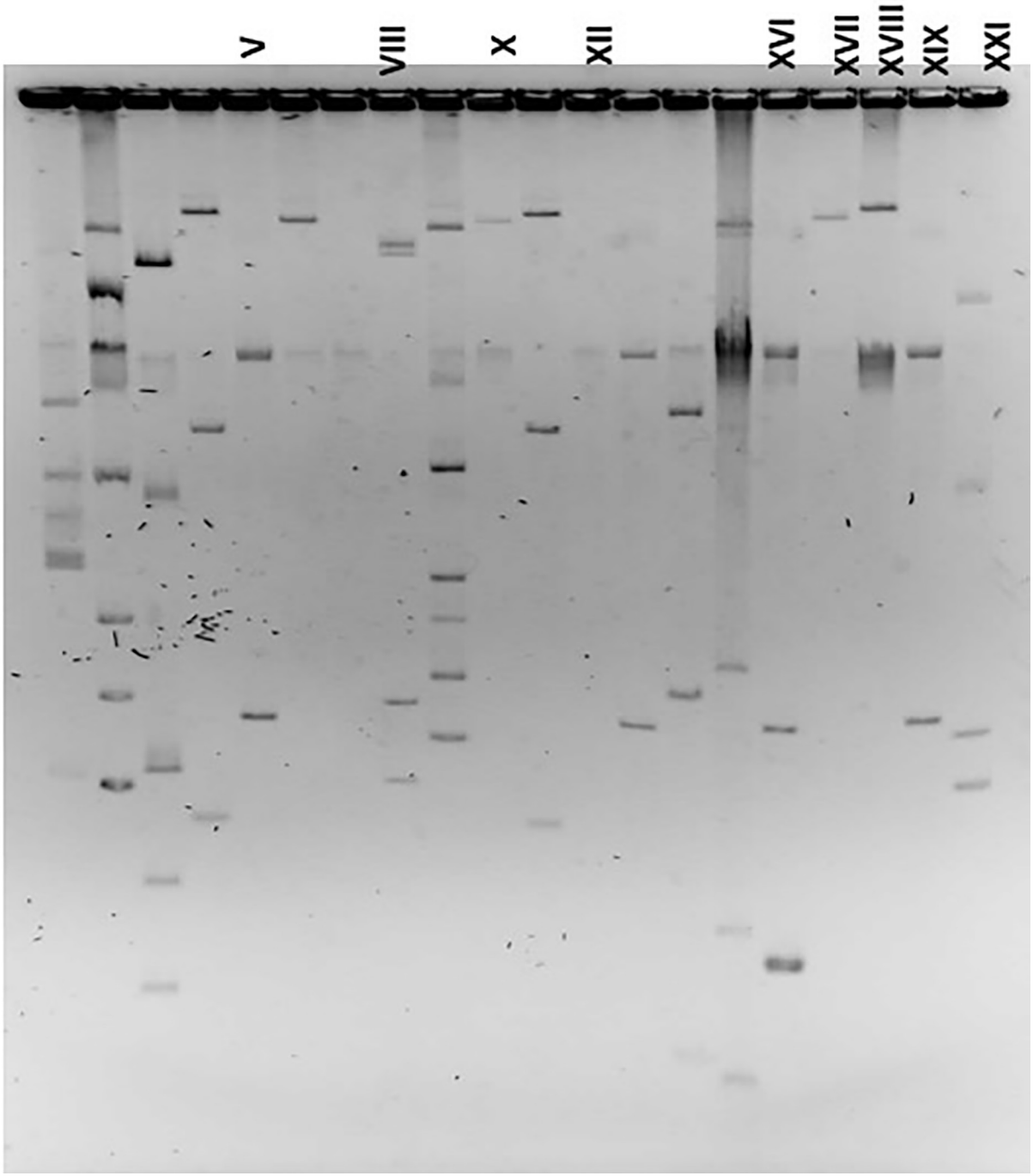

Fig 2. Plasmid profiles of *Acinetobacter* strains. Lanes marked with roman numerals are plasmid profiles isolated from *Acinetobacter baumannii*. Unmarked lanes are plasmid profiles of other *Acinetobacter* species isolated during this study period. Plasmid profile (strain number): V (2161), VIII (2339), X (3108), XII (3496), XIII (5189), XVI (4197), XVII (6793), XVIII (2275), XIX (2663), XXI (3275).

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were on mechanical ventilation, 37 (56.1%) developed VAP, with a median of 8 days (IQR 3, 20 days) from intubation until *A. baumannii* isolation. Table 2.

Twenty-one patients (19.8%) died within 72 hours after *A. baumannii* isolation; 20 (95.2%) were classified as infected. During the first month, 53 patients (50%) died: 8 (20%) in the

Table 1. Demographic and general characteristics of 106 patients with MDR- *A. baumannii*, classified as colonized or infected.

Characteristics N (%)	Total–N = 106	Colonization–n = 40 (37.7)	Infection–n = 66 (62.3)	*P*
Age (years)[a]	48.3 ± 16	51.1 ± 16	46.5 ± 15.9	0.156
Male gender	59 (55.7)	21 (52.5)	38 (57.6)	0.610
Solid tumor	54 (50.9)	28 (70)	26 (39.4)	0.002
Hematologic malignancy	52 (49.1)	12 (30)	40 (60.6)	
Oncologic status				0.393
Recent diagnosis	66 (62.3)	23 (57.5)	43 (65.2)	
Progression	13 (12.2)	5 (12.5)	8 (12.1)	
Relapse	12 (11.3)	4 (10)	8 (12.1)	
Complete remission	9 (8.5)	6 (15)	3 (4.5)	
Partial remission	6 (5.7)	2 (5)	4 (6.1)	
Recent chemotherapy[b]	44 (41.6)	14 (35)	30 (45.5)	0.289
Recent radiotherapy[c]	10 (9.4)	2 (5)	8 (12.1)	0.311
Previous hospitalization[b]	55 (51.9)	20 (50)	35 (53)	0.762
Median length of previous hospitalization[d] (days)	10 (5,17)	9 (4, 218)	10 (5,15)	0.881
Recent use of antimicrobials[b]	26 (24.5)	7 (17.5)	19 (28.8)	0.048
Severe neutropenia	16 (15.1)	5 (12.5)	11 (16.7)	0.780
Major surgery	28 (26.4)	16 (40)	12 (18.2)	0.013

[a]Mean ± standard deviation.

[b]During the previous three months.

[c]During the previous 6 months.

[d]Median (IQR).

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colonized group and 45 (68.2%) in the infected group ($p < 0.001$). (Fig 3). From the infected patients who died, 16/18 (88.9%) had BSI, 22/30 (73.3%) had pneumonia, and 7/18 (38.9%) had *A. baumannii* isolated from another site ($p = 0.003$). The time between *A. baumannii* isolation and death was shorter among patients whose strain was isolated from blood (median 3 days; IQR 2,5), compared with patients whose strain was isolated only from bronchial aspirates (median 8 days; IQR 3, 22), ($p = 0.03$).

Considering only the infected patients, 32 (48.5%) received an appropriate antibiotic against *A. baumannii*, of which 29 received colistin (four as monotherapy, fifteen combined with rifampicin, two with meropenem, eight with meropenem and rifampicin), the mean days of colistin were 12 ± 6 days (no differences between patients with BSI, with VAP or with other infection). Two patients received tigecycline (one as monotherapy and one combined with meropenem), for 7 and 14 days each one. Eighteen from 32 patients who received appropriate treatment died in the first 30 days (56.2%), compared with 27 from 34 patients (79.4%) who did not receive an appropriate treatment ($p = 0.06$).

In univariate analysis, infection vs. colonization, hematologic malignancies and inappropriate antimicrobial treatment were the risk factors significantly associated with 30-day mortality. In the multivariate regression analysis, infected patients and inappropriate antimicrobial treatment were the risks factors associated with death. Table 3.

Discussion

We describe an outbreak that occurred from 2011 to 2015 (92.4% of the strains with MLST 758), peaking during 2011 and 2012 in the ICU, but persisting during the following years (2013 to 2015), including strains from patients hospitalized in wards outside the ICU. The

Table 2. Clinical characteristics related to *A. baumannii* isolation in 106 patients, classified as colonized or infected.

Characteristics	Total	Colonization	Infection	P
	n = 106 (%)	n = 40 (37.7)	n = 66 (62.3)	
Site of *Acinetobacter* isolation				<0.001
Blood cultures	18 (17)	0	18 (27.3)	
Bronchial aspirate	49 (46.2)	19 (47.5)	30 (45.4)	
Surgical site infection	20 (18.9)	15 (37.5)	5 (7.6)	
Urine	12 (11.3)	3 (7.5)	9 (13.6)	
Other	7 (6.6)	3 (7.5)	4 (6.1)	
Hospital length before *A. baumannii* isolation (days)[a]	17.6 ± 13.6	21.6 ± 17.1	16.3 ± 12.51	0.136
ICU hospitalization[b]	66 (62.3)	20 (50)	46 (69.7)	0.046
ICU stay before *A. baumannii* isolation (days)[a]	10.1 ± 8.9	12 ± 11.4	9.3 ± 7.7	0.297
ICU whole length stay (days)[a]	15.9 ± 12.6	15.8 ± 12.5	16 ± 12.8	0.931
SOFA at ICU admission[acd]	8 (5, 11)	7 (5, 10)	8 (6, 11)	0.344
SOFA at *A. baumannii* isolation	3 (0, 9)	0 (0, 2)	7 (3,11)	<0.001
Mechanical ventilation (MV)	66 (62.3)	19 (47.5)	47 (71.2)	0.01
Mean length of MV (days)[a]	15.2 ± 12.5	15.8 ± 12.4	15 ± 12.7	0.828
A. baumannii isolated from bronchial aspirate after 48 h of MV[e]	37 (56.1)	14 (73.7)	23 (48.9)	0.100
Days of MV until isolation[a]	12.6 ± 9.2	15.6 ± 12	10.8 ± 6.7	0.128
Days from *A. baumannii* isolation until discharge[d]	8 (3, 20)	7 (4, 14)	9 (3, 22)	0.154
Mortality at 72-hours	21 (19.8)	1 (2.5)	20 (30.3)	<0.001
Mortality at 30-day	53 (50)	8 (20)	45 (68.2)	<0.001

[a]Mean ± standard deviation.

[b]ICU: Intensive Care Unit.

[c]SOFA (Sequential Organ Failure Assessment) at ICU admission were calculated in 66 patients: 20 colonized and 46 infected.

[d]Median (interquartile range).

[e]Percentage was obtained from the total of patients with mechanical ventilation.

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strain was introduced into the institution by a patient (index case) with non-Hodgkin lymphoma transferred from another clinic outside Mexico City. MDR *A. baumannii* was isolated at arrival from the insertion site of a pleural tube. Initially, it was considered as a contaminant, and no treatment was prescribed. One week later, he developed respiratory failure, required mechanical ventilation, was admitted to the ICU and broad-spectrum antibiotics were administered. Three weeks after admission he received chemotherapy and seven days later he developed febrile neutropenia and MDR *A. baumannii* bacteremia was documented. He received colistin for 14 days and was transferred to another hospital still with clinical signs of serious infection. It has been demonstrated that *A. baumannii* is able to overcome specific and general host defense systems, and survive in contact with human host fluids and tissues [16].

In this series, we included 99 patients with an MLST 758 strain: 95 with the same plasmid profile (V), two with plasmid XVI- very similar to V, and two with another plasmid profile. The remaining seven isolates belonged to different MLSTs with different plasmids patterns; all of them have been hospitalized recently. They were probably colonized with the MDR strain when they arrived, and it became invasive during hospitalization due to therapeutic procedures.

This MLST has been identified previously in Mexican hospitals and in other countries of Latin America [17–19]. The MLST758 is an understudied lineage that does not belong to the international clones I and II; it has spread to Canada, Mexico, Honduras, and Colombia. This lineage represents a source of genetic diversity of MDR and XDR isolates that have not been studied [20, 21].

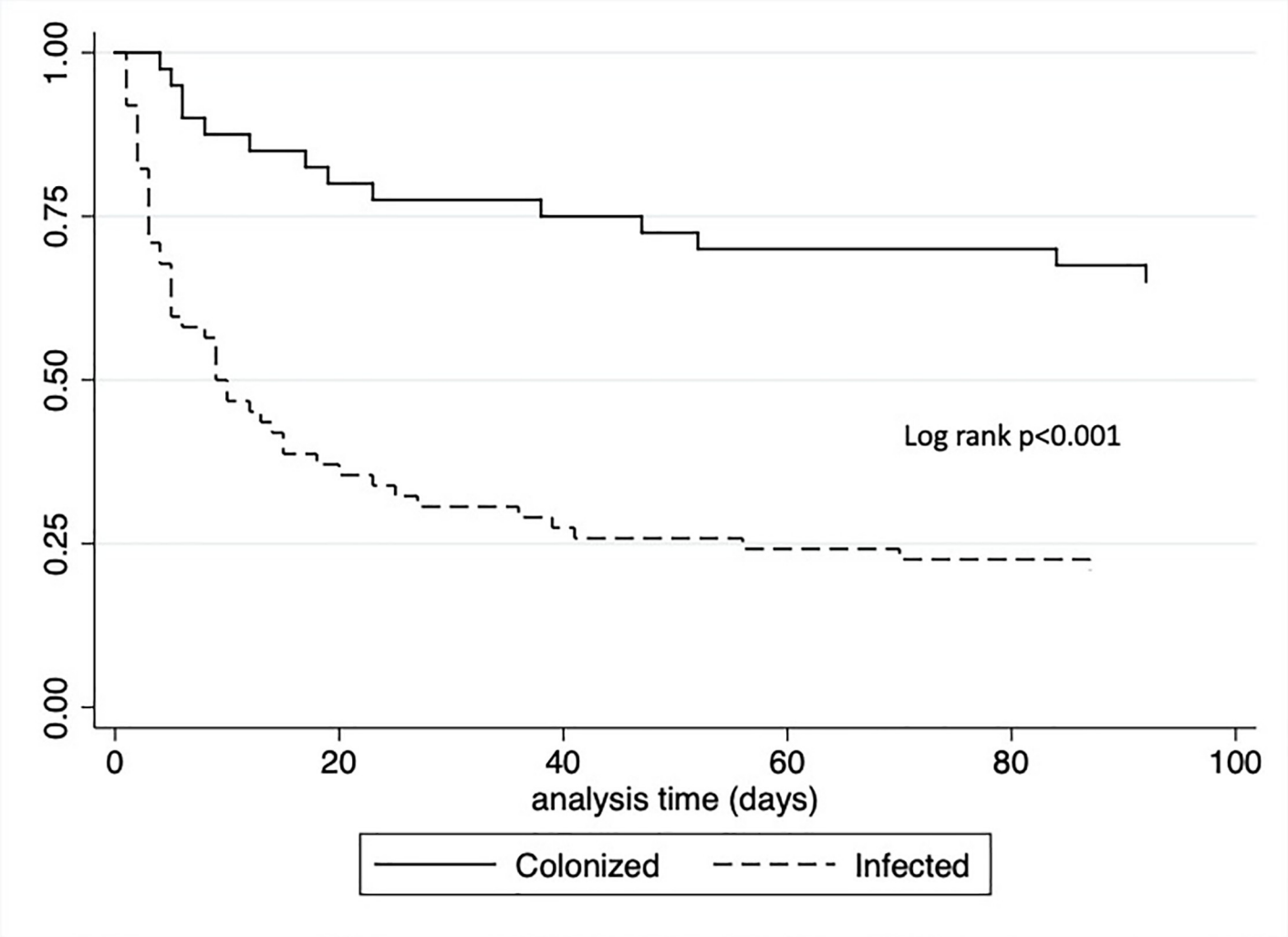

Fig 3. Kaplan Meier survival in 106 patients with *A. baumannii* divided by colonized (n = 40) or infected (n = 66).

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Currently, colistin seems to be the most reliably effective drug in vitro against MDR *A. baumannii* [1, 3]. The efficacy of this antibiotic in severe infections caused by *A. baumannii* has been demonstrated in several retrospective and prospective series including patients with serious infections such as pneumonia and bacteraemia [3].

In this study, 32 of the 66 patients classified as infected who received appropriate antimicrobial treatment had lower mortality (56.2%), than those who did not receive appropriate antimicrobial therapy (79.4%), although it was not statistically significant. There were two reasons why patients did not receive appropriate treatment:1) 21 patients died within 48 hours after culture was taken, and MDR *A. baumannii* had not been reported yet; 2) the other thirteen died because colistin was not regularly available in our hospital during 2011 and the first half of 2012. This high mortality in the first hours is related to extremely rapid clinical progression described in cases with *Acinetobacter* infection [22].

The last strain (isolated in 2015) was from a patient that already was infected when he arrived from another hospital from the west coast of Mexico. The plasmid profile was similar to the outbreak strain; we presume it was reintroduced to our hospital but did not spread further.

Table 3. Uni- and multivariate analysis for 30-day mortality in patients MDR- *A. baumannii*.

Characteristics—*N* (%)	Alive (*n* = 53, 50%)	Death (*n* = 53, 50)	Univariate		Multivariate	
			OR (95% CI)	*P*	OR (95% CI)	*P*
Age ≤60 (years)	44 (83)	40 (75.5)	1.6 (0.55–4.68)	0.473	-	
Age ≥60	9 (17)	13 (24.5)				
Colonized	32 (60.4)	8 (15.1)	8.6 (3.12–24.86)	<0.001	1	<0.001
Infected	21 (39.6)	45 (84.9)			6.58 (2.34–18.5)	
Solid tumor	36 (67.9)	18 (33.9)	4.1 (1.7–10.1)	<0.001	1	0.03
Hematologic malignancy	17 (32.1)	35 (66.1)			2.12 (1.09–9.35)	
Recent diagnosis/CR[a]	40 (75.5)	36 (67.9)	1.45 (0.57–3.73)	0.388	-	
Progression/relapse	13 (24.5)	17 (32.1)				
No recent chemotherapy	33 (62.3)	29 (54.7)	1.36 (0.58–3.19)	0.43	-	
Recent chemotherapy[b]	20 (37.7)	24 (45.3)				
Appropriate treatment[c]	8 (15.1)	29 (54.7)	2.9 (0.89–96)	0.06	1	0.134
Non-appropriate treatment	13 (24.5)	16 (30.2)			2.03 (0.8–5.15)	
SOFA score <10[d]	48 (90.6)	37 (69.8)	3.78 (1.16–14.37)	0.02	1	0.525
SOFA score >10	5 (9.4)	16 (30.2)			1.51 (0.42–5.41)	

[a]Recent oncology diagnosis or complete remission.

[b]Chemotherapy in the previous month.

[c]Analysis was performed only in those patients considered as infected.

[d]SOFA score. Sequential Organ Failure Assessment calculated when MDR-*A. baumannii* culture was taken.

https://doi.org/10.1371/journal.pone.0234684.t003

MDR *A. baumannii* isolation has increased during the last decades. In the 1970s, the majority of strains were sensitive to the commonly used antibiotics, but by 2007, up to 70% of isolates in certain settings were MDR, including some with resistance to carbapenems. Resistance is usually due to combined mechanisms, commonly including cell membrane impermeability, increased expression of efflux pumps, and production of beta-lactamases, commonly OXA type [23]. Exposure of *A. baumannii* to the selective pressure of potent antimicrobials has gradually led to a global prevalence of strains that are resistant to all beta-lactams, including carbapenems [23].

Recently, employing a population genomics approach, we described the emergence and dispersion of the lineage ST758, and also studied the antibiotic resistance mechanisms present in strains from this ST. This lineage emerged rather recently (about 22 years ago) and since then it has spread in many countries in Latin and North America; notably, strains from this lineage are not only carbapenem resistant but also multidrug resistant. In terms of acquired oxacillinases (OXAs), this lineage presents the worldwide-distributed OXA-23-like family but also the less disseminated OXA-40-like family [24].

Outbreaks caused by MDR *A. baumannii* have been identified in several ICUs worldwide, and the majority only susceptible to colistin [23]. Risk factors for MDR *A. baumannii* infections have been identified: previous hospitalization (the longer the stay, the higher the risk), use of invasive devices, surgical procedures, immunosuppression, admission to the ICU, mechanical ventilation, previous use of antibiotics, and colonization by this bacterium [3, 25]. In our series, 46% of patients had been hospitalized during the previous three months, 28% had used broad-spectrum antimicrobials, 83% had been admitted to the ICU, with a mean length of 16 days in the ICU and a mean length of 18 days in the hospital prior to *A. baumannii* isolation.

During the outbreak, prevention measures were implemented and others were reinforced; the measures included: a single nurse per patient, a single cleaning material quit per each

room, exhaustive daily cleaning of all ventilation equipment, hand hygiene program reinforcement, strict contact isolation, chlorhexidine oral washing three times per day, and patient daily bathing with chlorhexidine. Antimicrobial control policies were implemented in year 2013. Also, for patients transferred from other hospital initial cultures from nares, axillary, perineal region and draining sites were taken. All the measures contributed to end the outbreak in year 2014. Environmental and tap water surveillance was also performed during the outbreak period (44 samples taken in 2011, and 111 in 2012). Only one tap water sample (isolated in Feb 2012) was positive for MDR *A*. *baumanniii* ST758 profile V at the hematopoietic stem cell transplant unit. No patient in this unit was diagnosed with MDR *A*. *baumanniii* infection.

Multiple studies have reported poor outcomes of MDR *A*. *baumannii* infection with high mortality rates and long hospital stays directly attributed to infection [26]. A study conducted in China found a crude and 30-day in-hospital mortality rate of MDR *A*. *baumannii* bacteremia of 21.2% and 12.7% respectively, lower than other reports in which high mortality rates ranged from 29% to 63.5%. This difference needs to be clarified as the severity of diseases and virulence factors are closely related to mortality [27]. A previous study carried out in Mexico reported 14.5% mortality [28]; this study included susceptible and resistant *A*. *baumannii* strains in a tertiary care teaching hospital; 22% of patients were not hospitalized in the ICU, which reflects less ill patients compared with this series. Mortality reported in this study was 20% in the first 72 hours, and 50% in the first month, in which patients with bacteremia had the highest mortality (89%). These results were similar to a retrospective cohort from Israel that reported increased mortality in patients with bacteremia and concomitant pneumonia [29]. As expected, the difference in mortality was significant when we compared colonized patients (20%) with infected patients (68.2%). Renal failure and SOFA score >7 have been documented as independent risk factors for mortality in the ICU [27, 30]. We did not find differences in 30-day mortality when SOFA scores were analyzed at ICU admission, but we found differences when this score was calculated the day when culture was obtained (SOFA ≥ 10 was calculated in 30.2% of patients who died in the first month, compared with 9.4% who were alive in the same period of time, $p = 0.02$).

Although we did not estimate costs, it is very important to consider the increase in costs for the care of MDR organisms outbreaks. In France, the costs observed in a 17-week outbreak was close to 500,000 US dollars [4].

The study has some limitations: we included patients from a single tertiary-care referral cancer center, that does not allow to extrapolate the data to other scenarios. We also were not able to get information from resistance isolates of the hospital where the index patient was hospitalized previous to the transfer to our institution; we were able to establish through molecular epidemiology the original source of the strain.

However, the strengths are that it allows establishing some risk factors associated with infection and mortality from this pathogen, and we were able to demonstrate the clonal relation in most all the strains of this outbreak.

Conclusions

The molecular epidemiology of this outbreak highlights the threat that represents the transfer of colonized patients with MDR strains from another institutions, and emphasizes the importance to adhere to strict preventive measures, particularly hand hygiene programs, and contact isolation in patients with MDR strains [30]. Although some of the strains collected had different plasmid patterns and did not belong to the main outbreak strain, they were obtained from patients that had been treated in other hospitals, which shows the constant menace of introducing MDR strains into a hospital.

Supporting information

S1 File. A method to obtain plasmid profiles from Acinetobacter.
(DOCX)

S1 Data.
(PDF)

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