The Plasmidome of Firmicutes: Impact on the Emergence and the Spread of Resistance to Antimicrobials

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ABSTRACT The phylum Firmicutes is one of the most abundant groups of prokaryotes in the microbiota of humans and animals and includes genera of outstanding relevance in biomedicine, health care, and industry. Antimicrobial drug resistance is now considered a global health security challenge of the 21st century, and this heterogeneous group of microorganisms represents a significant part of this public health issue.

The presence of the same resistant genes in unrelated bacterial genera indicates a complex history of genetic interactions. Plasmids have largely contributed to the spread of resistance genes among Staphylococcus, Enterococcus, and Streptococcus species, also influencing the selection and ecological variation of specific populations. However, this information is fragmented and often omits species outside these genera. To date, the antimicrobial resistance problem has been analyzed under a "single centric" perspective ("gene tracking" or "vehicle centric" in "single host-single pathogen" systems) that has greatly delayed the understanding of gene and plasmid dynamics and their role in the evolution of bacterial communities.

This work analyzes the dynamics of antimicrobial resistance genes using gene exchange networks; the role of plasmids in the emergence, dissemination, and maintenance of genes encoding resistance to antimicrobials (antibiotics, heavy metals, and biocides); and their influence on the genomic diversity of the main Gram-positive opportunistic pathogens under the light of evolutionary ecology. A revision of the approaches to categorize plasmids in this group of microorganisms is given using the 1,326 fully sequenced plasmids of Gram-positive bacteria available in the GenBank database at the time the article was written.

INTRODUCTION Firmicutes constitutes one of the dominant bacteria phyla of human and animal gut microbiota. It comprises a number of genera of outstanding relevance in health care and industry such as Staphylococcus, Listeria, and lactic acid bacteria (LAB), a group of microorganisms that ferment carbohydrates into lactic acid and that includes the genera Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, and

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Weisella. Furthermore, species of *Negativicutes* (*Selenomonas*, *Veillonella*) and *Clostridium* have clinical interest for humans and animals (Table 1).

Antibiotic resistance (AbR) in this heterogeneous group of organisms constitutes a significant part of the public health problem. The most recent report by the Centers for Disease Control and Prevention in the United States provides a ranking list of AbR human pathogens according to their threat level to society and the attention that such a problem requires. Gram-positive organisms were grouped in the categories of “urgent” (*Clostridium difficile*), “serious” (methicillin-resistant *Staphylococcus aureus* [MRSA], antibiotic-resistant *Streptococcus pneumoniae*, vancomycin-resistant *Enterococcus* [VRE]), and “concerning” (erythromycin-resistant *Streptococcus pyogenes* and clindamycin-resistant *Streptococcus pneumoniae*).

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<sup>a</sup>Nocardiacceae (4 Nocardia, 25 Rhodococcus)

<sup>b</sup>Mycobacteriaceae (Mycobacterium plus Amycoliciocus)

<sup>c</sup>Micrococcaceae (4 Micrococcus, 23 Arthrobacter)

<sup>d</sup>Pseudonocardiacceae (2 Amycolatopsis, 6 Pseudonocardia, 1 Saccharomonospora)

<sup>e</sup>Microbacteriaceae (4 Clavibacter)

<sup>f</sup>Streptosporangiacceae (1 Planobispora, 1 Streptosporangium)

<sup>g</sup>Promicromonosporaceae (Xylanimonas)
resistant *Streptococcus agalactiae*) on the basis of the limited therapeutic options to treat infections caused by these bacteria-resistant variants (1). LAB, which are used as probiotics and in the preparation of various products (dairy, fermented meat and seafood, fermented cereals and vegetables, wine), are defined as “generally regarded as safe” (GRAS) microorganisms by the U.S. Food and Drug Administration. However, the potential risk to transfer acquired AbR genes recently found in LAB species to animal and human pathogens is a cause for concern. AbR LAB may also contaminate industrial processes, leading to economic losses (2). In addition, the possibility that opportunistic or commensal bacteria and nonpathogen organisms could serve as reservoirs of AbR genes is increasingly recognized (3). Consequently, several European and American regulatory agencies have recently recommended the mandatory screening of some species such as *Enterococcus faecalis* and *Enterococcus faecium* as indicators of the presence of AbR in foods and food animals and as a mirror of the patterns of antibiotic use in veterinary medicine and agriculture (4, 5). Finally, it is worth mentioning that AbR in a context of the wide use of antibiotics favors the selection of clonal lineages of multihost species with zoonotic potential (e.g., *S. aureus*, *E. faecium*, *Clostridium perfringens*) as well as emblematic zoonotic species such as *Listeria monocytogenes* (see below).

The presence of the same AbR genes in ecologically connected (but also in unconnected) bacterial genera, mentioned above, indicates a complex history of genetic interactions in which AbR genes have parasitized the natural circuits of adaptive gene flow. Plasmids have largely contributed to the spread of AbR and other adaptive genes among members of *Staphylococcus*, *Enterococcus*, and to a lesser extent, species of the *Streptococcus* pyogenic group (6–8), thus influencing the selection of particular subspecies populations due to the acquisition of AbR (8–10). However, the global adaptive role of plasmids of other genera remains largely unexplored outside single pathogen colonizing or infecting single “relevant” hosts. The “single centric” perspective, focusing on “gene tracking” or “vehicle centric” (plasmid, transposon, or other mobile genetic elements [MGEs]) in “single host-single pathogen” systems hampers a comprehensive view of gene and plasmid dynamics and their role in the evolvability of bacterial communities. An integrative view of plasmid ecology is needed to understand community evolvability.

In this work, we analyze the development of AbR in *Firmicutes* within an ecological framework using gene exchange networks. We also discuss the role of plasmids in the emergence, spread, and maintenance of genes encoding resistance to antimicrobials (antibiotics, heavy metals, and biocides) and their influence on the genomic diversity of the main Gram-positive opportunistic pathogens in the light of evolutionary ecology. Finally, a critical revision of plasmid classifications in this group of microorganisms is also provided under this eco-evo perspective by analyzing the 1,326 fully sequenced plasmids of Gram-positive bacteria (*Firmicutes* and *Actinobacteria*) available in the GenBank database at the time this article was written.

AN ECO-EVO PERSPECTIVE TO ANALYZE HGT IN FIRMICUTES

Recent phylogenomic analyses using networks revealed a history of horizontal gene transfer (HGT) events even among highly structured and ecologically disconnected groups of bacteria (11–13). These events are more likely to occur in the case of donors and recipients with a similar G+C content (differing in <5% for 86% of connected pairs) (14) and involving plasmids able to mediate exchange of information between close or distant chromosomal backgrounds (12, 15). Although limited by the current number of available genome sequences, such studies evidenced sound differences in “betweenness” among different bacterial groups and plasmids of *Firmicutes*. LAB frequently undergo HGT events among similar species (11), with streptococci acting as a hub for interactions with more distant ecological groups (12), and some plasmids of the Inc18 family possibly contributing to the spread of AbR genes among different bacterial species (15). To analyze this situation in more detail, we constructed a gene exchange network that comprises all genes conferring resistance to antibiotics and heavy metals described in *Firmicutes* so far (Fig. 1 and 2). This network clearly shows that many resistance genes in different bacterial genera can present plasmid and/or chromosomal locations, illustrating the diversity of interactions, often plasmid mediated, within bacterial communities (Fig. 1 and 2). Available (and often fragmented) knowledge from different fields enabled us to state that the dynamics of bacterial populations are influenced by the interplay of selection processes at different levels of organization (genes, MGEs, clones, species) and their associated environments (16–20). Because of that, the complexity resulting from such interplay cannot be understood using either single centric studies or the above-mentioned phylogenomic analysis of HGT networks.

The presence of the same genes in different genetic contexts implies contacts and exchanges between bac-
FIGURE 1  Protein content network (PCN) of AbR proteins found in plasmids and chromosomes of Firmicutes and Actinobacteria. To determine the AbR protein catalog of Gram-positive strains (chromosomes and plasmids), a Blastp search was performed of all their proteomes against the ARG-ANNOT database (http://en.mediterranee-infection.com/article.php?laref=283&titre=arg-annot) using a cut-off of 1e-30 and 85% of identity. The presence of the Gram-positive AbR proteins identified above in all bacterial species (only complete sequences, not partial) was determined using a similar Blast search (blastp, 1e-30 E-value and 85% identity) against the NCBI GenBank database. The nodes correspond to bacterial species (circular nodes; each color indicates one genus) and AbR proteins (square nodes). Nodes were connected by an edge when a positive hit between AbR proteins and one or more strains of a given species were identified. Edges further indicate the location of the AbR genes associated with each AbR protein of the Gram-positive catalog. Solid lines represent chromosomal location, and dotted lines represent plasmid location. When an AbR gene was located in both chromosomes and plasmids, both lines were plotted. doi:10.1128/microbiolspec.PLAS-0039-2014.f1


The Plasmidome of Firmicutes

Efforts in plasmid characterization and classification are justified for the understanding of plasmid biology. Nowadays, plasmid categorization is relevant from the public and environmental health perspective to follow the movement of genes coding for resistance to antimicrobials (antibiotics, heavy metals, biocides), colonization and virulence factors for humans and animals, and/or other adaptive traits that drive ecological success (bacteriocins, metabolic traits) and consequently increase the population size of bacteria harboring MGEs. In fact, only a “representative diversity” of bacterial plasmids has been systematically analyzed in a few genera of multihost opportunistic pathogens of interest in biomedicine, with a particular emphasis on species of the Enterobacteriaceae, Pseudomonadaceae, Staphylococcaceae, and Enterococcaceae families (7, 50–53).

The diversity of plasmids from Lactococcus (54), Lactobacillus (55), C. perfringens (56), Micrococcus (57), and Bifidobacterium (58) has also been analyzed from different perspectives.

Plasmid diversity within a particular bacterial species in the Firmicutes phylum started to be comprehensively analyzed in the 1960s just after the discovery of staphylococcal plasmids. These elements were initially categorized into three main classes designated by roman numerals on the basis of size, replication machinery,
ability to be transferred, phenotypic and functional characteristics, and host range (7, 51, 53, 59, 60). Class I comprised high copy number plasmids (10 to 60 copies per cell) of less than 5 kb with a rolling circle replication (RCR) mechanism that often harbored one or two AbR genes (usually conferring resistance to tetracycline, chloramphenicol, macrolides, and trimethoprim). Class II comprised low copy number plasmids (4 to 6 copies per cell) of 15 to 40 kb, with a theta replication mechanism, which typically carried resistance to antibiotics (β-lactams, aminoglycosides, and macrolides), heavy metals (arsenic, cadmium, and mercury), and/or antibiotics (quaternary ammonium compounds). Class III comprised plasmids similar to those found in class II which were transferred by conjugation (61). Afterward, Richard Novick and others classified staphylococcal plasmids in 15 incompatibility (Inc) groups based on the finding that two plasmids with the same replication (rep) proteins cannot be stably maintained in the same cell (50, 62, 63). Plasmids of most Inc groups correspond to class I (10 Inc groups of apparently closely related plasmids) and class II (diverse plasmids that belong to the same Inc group) (53). Following the same Inc numerical designation criteria, Brantl et al. categorized a few streptococcal plasmids that replicated via a theta mechanism and that were regulated by an antisense RNA that mediated transcriptional attenuation, such as the Inc18 family (64) (see below). Pheromone-responsive plasmids of enterococci were also subgrouped into different incompatibility groups on the basis of distinct responses to small peptides or pheromones which are secreted by plasmid-free donors (65).

A multiplex-PCR typing system based on the diversity of replication initiator proteins (RIPs) developed by Jensen et al. (59) has recently been applied for the characterization of Firmicutes plasmids, mainly staphylococci (66) and enterococci (67–71) of human, animal, and environmental origin. According to this typing system, RIP variants are designated as “rep” followed by a subindex number and are arbitrarily called Rep families. Although this system is very useful to enlarge the knowledge of scarcely explored plasmid diversity in contemporary isolates of enterococci and staphylococci, its application is limited to known plasmids, mainly AbR plasmids of these genera, as illustrated in various surveys and this study (59, 66, 68, 71).

The diversity of mobilization (MOB) systems has also recently been used to classify plasmids and other conjugative elements in different bacterial groups including Firmicutes (72, 73). The approach relies on the variability of relaxases (RELs), which form part of the plasmid MOB region, are involved in the initiation of DNA transfer, and that, aside from the origin of transfer (oriT), are present in both conjugative and mobilizable plasmids as well as in other conjugative elements (74). To date, only five (MOBP, MOBQ, MOBV, MOBC, and MOBF) out of seven known REL families have been identified in Firmicutes (7, 72, 74). MOBQ, MOBC, and MOBF are present in conjugative elements, and MOBV is present in mobilizable plasmids. MOBP has been identified in both conjugative and mobilizable elements (7, 72, 73). The application of this PCR-based classification scheme is obviously limited to the typing of known RELs. Frequent plasmid mosaicism, redundancy, and coexistence of different “core” genes, and the interplay of plasmids with other conjugative elements that contain homologs of RIPs and RELs, complicates the establishment of a robust plasmid core ontology and precludes the use of typing approaches similar to those used in Gram-negative organisms such as plasmid multilocus sequence type (http://pubmlst.org/plasmid/).

Whole-genome (plasmid/chromosome) sequencing provides accurate and nonbiased information on plasmid backbones. Although the number of fully sequenced

**FIGURE 2** PCN of metal-biocide (Met⁹/Bc⁸) proteins found in plasmids and chromosomes of Firmicutes and Actinobacteria. To determine the Met⁹/Bc⁸ protein catalog of Gram-positive strains (chromosomes and plasmids), a Blastp search was performed of all their proteomes against the BacMet database (http://bacmet.biomedicine.gu.se/) using a cut-off of 1e-30 and 85% of identity. The presence of the Gram-positive Met⁹/Bc⁸ proteins identified above in all bacterial species (only complete sequences, not partial) was determined using a similar Blastp search (blastp, 1e-30 evalue and 85% identity) against the NCBI GenBank database. The nodes correspond to bacterial species (circular nodes) and Met⁹/Bc⁸ proteins (triangular nodes). Nodes were connected by an edge when a positive hit between Met⁹/Bc⁸ proteins on one or more strains of a given species was identified. Edges further indicate the location of the Met⁹/Bc⁸ genes associated with each Met⁹/Bc⁸ protein of the Gram-positive catalog. Solid lines represent chromosomal location, and dotted lines represent plasmid location. When a Met⁹/Bc⁸ gene was located in both chromosomes and plasmids, both lines were plotted. doi:10.1128/microbiolspec.PLAS-0039-2014.12
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plasmids in databases is still limited, we used a plasmid homology network analysis of the 1,326 fully sequenced plasmids of Firmicutes and Actinobacteria to study the diversity of plasmids carrying genes coding for AbR and MetR/BcR and the impact of plasmids in the evolvability of contemporary AbR bacterial populations of Firmicutes. 

Figures 3 and 4 illustrate the existence of group-specific plasmid populations, with a number of plasmids being shared between Lactobacillales (mainly Enterococcus and Streptococcus) and Bacillales (Staphylococcus), which are greatly implicated in the spread of AbR and MetR/BcR. These shared plasmids include RCR and theta-replicating plasmids of different families, which have been recently analyzed at the molecular level (2, 75, 76). Next in this section, we will analyze the diversity of these groups and highlight the usefulness of current typing systems for each group. However, it is of note that the main genera of Firmicutes carry a variable number of plasmids containing several replication and transfer systems, some of them being able to be transferred. The interplay between genes, plasmids, and populations will be analyzed under an ecological perspective in the section “Gene and Plasmid Flow Shapes the Evolutionary Ecology of Firmicutes.”

**Rolling Circle Replication Plasmids**

RCR plasmids are classified in a few families according to the RIP and the double origin of replication (dso) (see comprehensive reviews in references 75, 77–79). Most of the RCR plasmids known to date have been found in species of Firmicutes, Proteobacteria, Cyanobacteria, and Spirochaetes, and some of them have been identified in genetically distant hosts. The production of single-stranded DNA and the mechanism of replication of these plasmids enhance their ability to recombine, by either homologous recombination or illegitimate recombination with other RCRs and theta replicating plasmids.

**FIGURE 3** Plasmid homology network. The genomic homology network was performed using “All-versus-All” genomic Megablast (238) of 1,326 fully sequenced plasmids from low G+C bacterial species (Firmicutes and Actinobacteria phyla) available at public gene databases. The nodes correspond to bacterial plasmids (circular nodes; different colors representing different genera). Two nodes are connected by an edge if they share homologous DNA. doi:10.1128/microbiolspec.PLAS-0039-2014.f3
RCR plasmids are also frequently integrated into chromosomes (e.g., pUB110 within SCCmec cassettes in methicillin-resistant *S. aureus* or *pC194/pUB110 [catA]* in *S. pneumoniae* genomes) (80).

In *Firmicutes*, four groups of RCR plasmids have been defined according to RIP similarity, namely Rep_trans (PF025486), Rep_1 (PF14046), Rep_2 (PF01719), and Rep_L, which are historically represented by plasmids pT181, pUB110, pMV158, and pSN2, respectively (53, 75, 77, 81). Within these families, some members have been fixed by selection and might be maintained by the vertical expansion of certain clones, aside from HGT, with the emergence of variants from time to time. Figures 11 to 14 and Supplementary Table S1 show the similarity of genes encoding RPs of all available fully sequenced plasmids and the correspondence to the Rep families described by Jensen et al. (59). These plasmids may contain different adaptive genes (AbR, heat shock proteins, or bacteriocins), although most of them are classified as “cryptic,” without any clear adaptive function.

### The Rep_1 family

The Rep_1 family comprises plasmids with RPs of the families rep13 (associated with *catA7*, which encodes resistance to chloramphenicol), rep21 (cryptic or eventually carrying *hnuA*, coding for resistance to lincosamides), rep22 (carrying a variety of AbR genes), and other underrepresented members categorized as rep_unique7. How-
FIGURE 5 Plasmids from Staphylococcus spp. The presence of an orange border in the RIP family indicates that the corresponding RIP is truncated. *PriCT_1; *One of these plasmids (GenBank accession number NC_013381) has a truncated rep_pKH21 (rep_1) gene, and no other known RIPS were identified. **Two of these plasmids (GenBank accession number NC_016054 and NC_019144) appear to have two copies of the MOB_Y gene. *One of these plasmids (GenBank accession number NC_008354) has two copies of the lnuA gene. †One plasmid (GenBank accession number NC_001393) has a truncated copy of the tetK gene. ‡One plasmid (GenBank accession number NC_010419) has a truncated copy of the blaZ gene. §The plasmid (GenBank accession number NC_005076) appears to have two copies of the MOB_Y gene. ‡One plasmid (GenBank accession number NC_018959) has a truncated copy of the blaZ gene. ‡Two plasmids (GenBank accession numbers NC_007931 and NC_016942) have two copies of the arsB and arsC genes. ‡This plasmid (GenBank accession number NC_013320) appears to have two copies of the MOB_Y gene. ‡This plasmid (GenBank accession number NC_005004) has a truncated copy of theblaZ gene. ‡Three plasmids (GenBank accession numbers NC_013321, NC_019007, and NC_018976) have a truncated copy of the blaZ gene. ‡Four of these plasmids have a truncated copy of the cadD gene (GenBank accession numbers NC_020531, NC_020538, NC_013337, NC_020534, NC_020565, NC_020567, NC_020530, NC_020539, NC_017352, NC_013323, and NC_022610). ‡This plasmid (GenBank accession number NC_002359) has a truncated copy of the cadD gene. ‡This plasmid (GenBank accession number NC_020237) appears to have two copies of the MOB_Y gene. ‡This plasmid (GenBank accession number NC_002598) appears to have two copies of the MOB_Y gene. Abbreviations: MRIP, Multi-RIP; S, Staphylococcus spp; Sar, Staphylococcus arlettae; Sa, S. aureus; Sc, Staphylococcus chromogenes; Se, Staphylococcus epidermidis; Sha, Shaemolyticus haemolyticus; Shy, Staphylococcus hyicus; Sle, Staphylococcus lentus; Slu, Staphylococcus lugdunensis; Sp, Staphylococcus pasteuri; Ssa, Staphylococcus saprophyticus; Ssc, Staphylococcus sciuri; Ssi, Staphylococcus simulans; Sw, Staphylococcus warneri. doi:10.1128/microbiolspec.PLAS-0039-2014.f5
ever, the available typing systems are unable to classify relevant Rep_1 plasmid members including plasmids containing heat shock proteins in *Streptococcus thermophilus*, plasmid-borne bacteriocins in *S. pyogenes* (82), or *S. pneumoniae* plasmids (80, 83), among others (Fig. 11). Remarkably, RPs of this Rep_1 group are often detected in mosaic plasmids of staphylococci and enterococci (Fig. 5 and 7), some plasmid chimeras being fixed and persistently recovered for years. For example, emblematic mosaic theta/RCR plasmids of staphylococci (e.g., cointegrates of Rep_A_N/pSK41 and Rep_1/pUB110, which encode resistance to gentamicin) and *E. faecalis* (e.g., pPAMa1) have both been selected in those lineages since the early 1970s (7, 71, 84).

The Rep_trans group
Plasmids of the Rep_trans group are clustered in two large branches (Fig. 12). One branch comprises plasmids of *Staphylococcus* that harbor tetK (rep Trans) and catA8/catA7 (rep Trans) with different MOB genes. Such plasmids have been reported in *S. aureus* since their first detection in the early 1950s (84) and were eventually described in contemporary *E. faecalis* isolates (68, 85). A second branch contains pRII-like plasmids (rep 1), which correspond to plasmids of different enterococcal species (*E. faecium*, *Enterococcus hirae*, *Enterococcus mundtii*) isolated from foodborne animals and hospital patients (7, 59, 71, 86). These plasmids can be mobilized by other AbR conjugative theta replicating plasmids present in the same cell (71, 87), and it seems they are widely spread among enterococcal populations.

The Rep_2 group
The Rep_2 group (Fig. 13) comprises numerous promiscuous elements able to replicate in distant hosts which have been extensively analyzed at the molecular level by Espinosa et al. using pMV158 as a model (75). Plasmids carrying ermT (an inducible methylase conferring resistance to first-line macrolide-lincosamide antibiotics such as erythromycin and clindamycin), from group A Streptococci (GAS) and group B Streptococci (GBS), are the sole representatives of AbR in this group. They appear to be responsible for the rise of macrolide resistance among GAS and GBS in hospitals since the mid-1990s (8).

The Rep_L group
In contrast to the above-mentioned RCR plasmid groups, proteins within the Rep_L family (Fig. 14) are represented in public gene databases by a very few RPs of *Staphylococcus*, *Selenomonas* (class Negativicutes), and *Butyryrivibrio* (Clostridia) species, all these genera being frequent components of the oral flora of humans and the rumen of some animal species. These plasmids are responsible for the widespread *ermC* in staphylococci (rep 10). Interestingly, the emergence of both *ermT*-Rep_2 and *ermC*-Rep_L plasmids seems to be associated with the abusive use of tylosin in cattle, amplified by the location of these AbR genes in RCR plasmids, and further transferred to other populations of *Firmicutes* (8, 88, 89).

RCR plasmids were associated with REL of the group MOBV1 (72, 73, 75), although representatives of all the RCR groups mentioned above that lack REL were detected in databases. Interestingly, RELs of MOBp1 and MOBf families were also found, and their presence is probably due to the co-integration of RCR with theta-replicating plasmids (see below).

**Theta-Replicating Plasmids**
Four families of plasmids that replicate by a theta mechanism, three that comprise conjugative plasmids (Rep_A_N, Inc18, and pMG1) and one in groups small nonconjugative elements (Rep_3), are involved in the capture, spread, and maintenance of AbR among different genera of *Firmicutes*. Members of the RepA_N and Inc18 families are often enriched in insertion sequences, mainly IS257, IS256, IS1216, ISL3, and IS431, that facilitate co-integration, rearrangements, and deletions among elements of *Staphylococcus*, *Enterococcus*, LAB, and *Clostridium* of different origins (6, 7, 28, 90–95). Such recombination events seem to have facilitated the origin of the great mosaicism of MDR plasmids that often carry more than one RIP, lack transfer and maintenance modules, and eventually carry more than one REL (Fig. 5 and 7). The transfer mechanisms of RepA_N pSK41-like plasmids and the Inc18-like plasmids are similar and are categorized as type IV secretion systems (96).

**The Rep_3 family**
Plasmids containing RPs with the Rep_3 domain (Fig. 15) are common among disparate bacterial genera including *Staphylococcus*, *Enterococcus*, *Streptococcus*, *Lactobacillus*, and *Enterobacteriaceae* (2). Figure 15 shows the diversity of RPs among fully sequenced plasmids of *Firmicutes*, and Fig. 5 to 10 reflect the features of known members of this family within each genus of biomedical interest. In enterococci, Rep_3 plasmids (<15 kb) have been found in isolates recovered from hospitalized patients, animals (pigs, cows), cheese, milk, and dry-fermented sausage, frequently associated with the production of bacteriocins that are active against a
variety of Gram-positive genera (7). In *Lactobacillus* and *Lactococcus*, they harbor bacteriocins and, eventually, AbR genes. Rep3 plasmids play a relevant role as vehicles of AbR among staphylococci. Plasmids from *S. aureus* are overrepresented by closely related variants containing Rep3, which are associated with genes coding for penicillinase and resistance to heavy metals (cadmium and arsenic) (51, 53, 66, 84). Staphylococcal plasmids within this group include AbR plasmids from coagulase-negative strains of animal origin, some of them with RIPs that would not be detected by current typing systems (97, 98).

The Inc18 family

First described in the 1990s, the Inc18 family comprises a highly heterogeneous group of broad host range, low copy number plasmids (<10 per cell) that replicate by theta mechanism, regulated by an antisense RNA that mediates transcriptional attenuation and that are able to conjugate on solid media at high frequencies (64, 99). The transfer system of pIP501 has been extensively studied and constitutes a paradigm of conjugation systems, showing significant similarity with the tra regions of RepA-N plasmids pGO1 and pSK41 from *S. aureus* and pMRC01 from *Lactococcus lactis* (96, 100).

The Inc18 group is traditionally represented by three emblematic plasmids: pSM19035 (101) and pIP501 from *S. agalactiae* and pAMβ1 from *E. faecalis* (64, 101–104). It gets its name from the apparent incompatibility of these plasmids with each other described in seminal studies in the field and following the nomenclature of Inc groups started by Richard Novick for staphylococcal plasmids (50, 60, 64, 105). Inc18 plasmids frequently carry the post segregational killing systems, εc, and type I partition cassette prgPprgO, which are associated with a variety of RIPs and seem to contribute to their persistence in different populations in the absence of antibiotic selection pressure (7, 106, 107). Detailed molecular characterization of such plasmids is described elsewhere (64, 99, 108) and shows a remarkably high modular interplay among different Inc18 plasmids, leading to the high modularity observed in plasmid sequences (see Fig. 5 to 10 and text below).

Inc18 plasmids have contributed remarkably to the spread of AbR (macrolides, chloramphenicol, aminoglycosides, and glycopeptides) and MetR (copper and mercury) among streptococci and other phylogenetically distant genera of Gram-positive (*S. aureus*, *Listeria*, *Bacillus subtilis*, *Lactobacillus*, *Leuconostoc*, various *Clostridium* species) and Gram-negative bacteria (108–111). Plasmid relatives of pAMβ1 (hARBoring *ermB*, and conferring resistance to macrolides, lincosamides, and streptogramines) and pIP501 (carrying *ermB* and *catA7pc221*, which confers resistance to chloramphenicol) were rapidly spread during the 1970s and have frequently been detected among streptococci of groups A, B, and D (enterococci) since then (110, 112–114) (see also Supplementary Table S1 for contemporary representatives of this plasmid group). Initially, the successful spread of intact AbR plasmids among clones of various streptococcal genera, including *S. pneumoniae*, and *S. aureus* was reported, despite the lack of stability in these last two clonal backgrounds (110, 113). Inc18 plasmids conferring resistance to aminoglycosides (kanamycin, streptomycin, and neomycin) and to macrolides were also detected in 1972, in the emblematic *Streptococcus* (*Enterococcus*) *fae- calis* strain JH1 that carried pJH1 (an MDR plasmid, presumably Inc18) and pJH2 (a RepA_N pheromone-responsive plasmid carrying hemolysin and bacteriocins). pJH1 represented the first description of conjugative transfer of AbR plasmids in enterococci (114). Aminoglycoside resistance in pJH1 relatives was due to the presence of Tn5405, a transposon comprising three genes in tandem (an aminoglycoside 6-adenyltransferase [aad], a streptothricin acetyltransferase [sat], and an aminoglycoside-phosphotransferase [aph3]). These genes were identified later on in *S. pyogenes*, *S. agalactiae*, *S. aureus*, *Campylobacter coli*, *C. perfringens*, and *C. difficile* (now *Peptoclostridium difficile*).

More recently, diverse Inc18 plasmids carrying Tn1546 in enterococci and staphylococci have emerged...
Plasmids from *Enterococcus* spp. The presence of an orange border in the RIP family indicates that the corresponding RIP is truncated. *Rep_2:* One of these plasmids (GenBank accession number NC_015849) has a truncated rep_AUS0004_p2 (Rep_1) gene, and no other known replication initiator proteins were found. **This plasmid (GenBank accession number NC_017962) has two copies of Tn401; in one of them the add(6) gene is not truncated; this plasmid also appears to have two copies of the MOBP1 gene.** +These two plasmids (GenBank accession numbers NC_008768 and NC_008821) have a truncated copy of the str gene. Abbreviations: MRIP, multi-RIP; Efm, *E. faecium*; Efc, *E. faecalis*; Emu, *E. mundtii*; Edu, *E. durans*; Ehi, *E. hirae*. doi:10.1128/microbiolspec.PLAS-0039-2014.f7
FIGURE 8 Plasmids from *Streptococcus* spp. \(^a\)Rep_trans; \(^b\)This plasmid (GenBank accession number NC_015219) has two similar replication genes belonging to the Rep_3 family. \(^c\)This plasmid (GenBank accession number NC_006979) has two similar replication genes belonging to the PriCT_1 family. Abbreviations: MRIP, Multi-RIP; Sag, *S. agalactiae*; Sdy, *Streptococcus dysgalactiae*; Sga, *Streptococcus galloylicus*; Sii, *Streptococcus infantarius*; Sln, *Streptococcus infantis*; Sma, *Streptococcus macedonicus*; Smu, *Streptococcus mutans*; Spa, *Streptococcus parasanguinis*; Spn, *S. pneumoniae*; Sps, *Streptococcus pseudopneumoniae*; SpG, *S. pyogenes*; Ssu, *Streptococcus suis*; Sth, *Streptococcus thermophilus*. doi:10.1128/microbiolspec.PLAS-0039-2014.f8
In different locations. In Europe, Inc18::Tn1546 plasmids (such as pVEF1, pVEF2, pVEF3, and pVEF4) seem to have evolved from pIP816 (the first Inc18::Tn1546 was isolated in France in 1987). They lack a transfer system and appear to be confined to E. faecium (70, 115, 116). Inc18::Tn1546 plasmids from the United States are linked to E. faecalis isolates (pWZ909, pWZ1668, pWZ1740) and contain a complete transfer system (117, 118). A plethora of multiresistant mosaic Inc18 plasmids containing up to three RPs, including RepR of pIP501 (CAA35647.1) and RepS of pRE25 (YP_783890.1), have been described in different Firmicutes (70, 71, 116, 119). These plasmids have an arsenal of insertion sequences, mainly IS1216 and ISL3, which facilitate genetic exchange with different genetic elements of different origins and the acquisition of different AbR (tetS) and MetR (tcrB, mer operon). These ISs also facilitate the co-integration with other RCR (e.g., pC221, which is co-integrated in pRE25) or theta replication plasmids as pheromone responsive (116, 119–121) or some pSK41-like elements. Figure 16 shows the diversity of Inc18 RPs that can be identified by typing systems. All these RPs have a primase domain PriCT_1 that allowed their identification as belonging to the Inc18 family. Fig. 6, 7, and 9 illustrate the mosaicism of Inc18 plasmids in enterococci and Listeria.

The pMG1/pHT plasmids

The pMG1/pHT plasmids are related to those of the Inc18 family (RP homolog approximately 30% identical to Inc18 initiators, including the PriCT_1 domain [Fig. 16]) (122), although they also show high homology with the pXO2 virulence plasmid from Bacillus anthracis. Because many open reading frames of pHT and pMG1 plasmids do not show significant homology with any reported proteins, they used to be categorized as a new type of highly efficient conjugative plasmids with a MOBp family REL. This plasmid group is represented by relatives of pHT (pHTα, pHTβ, and pHTγ) and pMG1, which have greatly facilitated the dissemination of resistance to glycopeptides (Tn1546_vanA) and high-level resistance to aminoglycosides (Tn4001-like elements) among human E. faecium and E. avium isolates from the United States and Japan (123, 124) and, to a lesser extent, European countries (7, 70, 71, 122).

The RepA_N family

This is a large family of plasmids (also including a few phages) that are widespread among the low G+C Gram-positive bacteria and which possess RIP homologs to the RepA protein of pAD1 (76). The five groups of RepA
homologs identified are phylogenetically congruent with their host background (Fig. 17), suggesting that the replicons have evolved along with their current hosts and that intergenus movement of RepA_N plasmids does not often occur. Such RepA_N clusters correspond to plasmids from *Staphylococcus* (Met^{R}/bla pSK1 and pSK41 MDR plasmids), plasmids from *Enterococcus* (*E. faecalis* pheromone-responsive plasmids and *E. faecium* non-pheromone-responsive plasmids related to pRUM, pLG1, or untypeable megaplasmids), plasmids from *Lactobacillus* and *Lactococcus*, phage homologs from *Streptococcus* (*S. pneumoniae, S. thermophilus*), and plasmids from *B. subtilis* (e.g., pLS32). Staphylococcal and enterococcal RepA_N plasmids have greatly contributed to the spread of AbR genes among humans and, eventually, animals and will be further described below. They also facilitate the movement of other non-conjugative plasmids and large genomic regions (36, 125, 126).

### RepA_N staphylococcal plasmids (Fig. 5)

Large staphylococcal MDR plasmids use evolutionarily related theta-mode replication, although they can be further divided into three types: the Met^{R}/beta-lactamase-producing plasmids, the pSK1 family, and pSK41-like conjugative elements. All these are compatible and can be identified as the rep_{19}, rep_{20}, and rep_{15} families, respectively, according to Jensen’s plasmid typing system (59, 127, 128). The pSK41 family (rep_{15}) is the largest group of conjugative plasmids in staphylococci, traditionally represented by pSK41, pG01, and pJE1, which emerged in the early 1980s associated with resistance to gentamicin due to the presence of Tn^4001 (84, 129). They often confer resistance to other antibiotics such as neomycin, tobramycin and kanamycin (due to the integration of pUB110 plasmids that harbor the aadD gene), antiseptics (due to the presence of qac genes) (130), and eventually trimethoprim (mediated by Tn^4001), penicillins (due to the presence of Tn552::blaZ), and others. Plasmids of this group may also confer resistance to mupirocin (131–133) and vancomycin (134, 135), represented by pUSA03 (which harbors ileS and tetK) and pWL1043 (which contains Tn1546, Tn4001, Tn4002, Tn552, and qacC). The pSK41-like plasmids are able to mobilize other plasmids present in the same bacterial cell (133, 136, 137). The pSK1 and Met^{R}/beta-lactamase plasmids belong to the same incompatibility groups and are also compatible with pSK41 plasmids. Despite their inability to self-transfer, these groups of plasmids have been detected in many staphylococcal species.
RepA_N enterococcal plasmids
This cluster groups pheromone-responsive plasmids of *E. faecalis* and pRUM- and pLG1-like plasmids of *E. faecium* (7) (Fig. 6 and 7).

Pheromone-responsive plasmids. Pheromone-responsive plasmids represent a paradigm of elements in the biology of MGEs and are, together with Inc18 plasmids, the best-known plasmids described to date. For details about the mechanism of replication, conjugation, and evolvability of this plasmid group see references 7, 49, 65, 92, and 138. Plasmids that respond to pheromones are present in most contemporary *E. faecalis* isolates from humans and birds but are only occasionally found among *E. faecium*. Synthesis of pheromones is confined to *E. faecalis*, although *Enterococcus hirae*, *S. aureus*, and *Streptococcus gordonii* may secrete cAM373-like peptides that facilitate the conjugation of pAM373 from *E. faecalis* (139). The description of cAM373-responsive plasmids coding for resistance to glycopeptides (Tn1546-vanA) highlights the potential risk of the spread of glycopeptide resistance in staphylococci in institutions where VRE are endemic (134, 140). Although pheromone plasmids are unable to replicate in *S. aureus*, their transference and establishment in this host might occur by co-integration with other plasmids able to replicate in this species. In addition, some pAD1 relatives enhance the rate of mobilization of plasmids, conjugative transposons, and PAs (125).

Plasmids of this family can be classified on the basis of responses to pheromones in different incompatibility groups (139) or according to RIP diversity (59, 68) within rep8 (pAM373) and rep9 (further split into subgroups rep9a(pAD1) and rep9b(pTEF2) families (59, 68). Transfer systems of MOBC or MOBP families have been detected in plasmids of this family.

Pheromone-responsive plasmids may encode putative virulence traits (aggregation substance, hemolysin/bacteriocin) and a diversity of AbR elements located on transposable elements such as Tn916-like (tetM), Tn4001 (aac-apb), Tn1546 (vanA), Tn1549 (vanB), and a composite transposon containing a β-lactamase gene flanked by two IS4 copies (7). The par locus encodes a unique antisense-regulated toxin-antitoxin system present in the plasmid pAD1, but par homologs have been detected on other plasmids and chromosomes of *E. faecalis* and *Staphylococcus*, *Clostridium*, *Listeria*, and *Lactobacillus* species (141). Toxin-antitoxin systems associated with other plasmid families such as ε2 and relBE have been detected on members of this plasmid group, reflecting rearrangements with representatives of other plasmid families (7). Even though to date, only a few members of pheromone-responsive plasmids have been fully sequenced, typing surveys reveal a wide diversity of plasmids among populations, often containing RIPs, RELs, or regions from plasmids of different origins (68, 71).

pRUM-like plasmids. pRUM-like plasmids (represented by pRUM, pS373c, pS177, and pDO2) are mosaic plasmids of variable size (>30 kb) that comprise diverse genetic elements of different origins (transposons, insertion sequences, small theta-replicating plasmids, bacteriocin clusters). They can be identified as the rep17 family according to PCR-based typing systems (59) but differ in the RIP sequence, the MOB system, and the presence of the toxin-antitoxin Axe-Txe locus (71, 142, 143). Both Inc18 and pRUM plasmids are driving the spread of glycopeptide resistance among contemporary isolates of *E. faecium* by carrying Tn1546 (vanA) or Tn1549 (vanB). Two types of pRUM plasmids are currently widespread among VRE and vancomycin-susceptible *E. faecium* isolates from different hosts. One contains RepA and Axe-Txe from pRUM and, eventually, the mobilization system of pC223 from *S. aureus* (70, 71, 142–144). The other type is characterized by a RepA protein that is 95% identical to RepA-pRUM, lacking postsegregational killing Axe-Txe and the presence of a MOBε relaxase originally detected in pEF1, a plasmid with an environmental origin. Tn1546 is frequently located on both types of pRUM plasmids, frequently containing replicons of other plasmid families (author’s unpublished results).

**FIGURE 11** Similarity of *rep*-like sequences encoding RIPs of the Rep_1 family. A neighbor-joining tree of gene sequences coding for RIPs of the Rep_1 family was built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on 1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylology/clustalw2_phylogeny/), and sequences showing an identity equal to or higher than 80% were clustered in groups that were highlighted by different backgrounds colors. Black dots indicate the RIP of the plasmid used for further comparison in Figs. 5 to 10. doi:10.1128/microbiolspec.PLAS-0039-2014.f11
FIGURE 12  Similarity of rep-like sequences encoding RIPs of the Rep_trans family. A neighbor-joining tree of gene sequences coding for RIPs of the Rep_trans family was built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on 1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/), and sequences showing an identity equal to or higher than 80% were clustered in groups that were highlighted by different backgrounds colors. Black dots indicate the RIP of the plasmid used for further comparison in Figs. 5 to 10. *Truncated gene. **Similar to E. faecalis ant6-la and aade. Abbreviations: ND, not determined. doi:10.1128/microbiolspec.PLAS-0039-2014.f12
**Large plasmids.** Plasmids larger than 150 kb are widely distributed among *E. faecium*, *Enterococcus durans*, and *E. hirae* from different origins, but they have not been detected among *E. faecalis* (71, 144–150). To date, only a handful of *E. faecium* megaplasmids have been fully sequenced (AUS0085_p1 [NC_021987], pNB2354_1 [NC_020208], DO_3 [NC_017963], and pLG1, although this last one has not been closed [148]). All of them contain a RIP similar to RepApAD1, making them part of the RepA_N family (Fig. 7, Supplementary Table S1) (59, 71, 148). A similar RIP has also been found in a 130-kb plasmid (NC_021987) from a VRE ST203 *E. faecium* strain isolated in 2009 in Australia (151). Although RIP sequences of pLG1 plasmids are often identified among enterococcal megaplasmids, most of them do not hybridize with known RIP genes included in published schemes (71, 148, 152). Enterococcal megaplasmids carry genes involved in sugar metabolism (mannitol, glycerol, sorbitol, raffinose, complex carbohydrates), AbR (macrolides, glycopeptides, aminoglycosides), MetR (copper-terYAZB), and enhanced adhesion (71, 126, 144, 147–149, 152–154). They are frequently involved in the acquisition or persistence of AbR among *E. faecium* isolates from food animals (144, 150).

**FIGURE 13** Similarity of rep-like sequences encoding RIPs of the Rep_2 family. A neighbor-joining tree of gene sequences coding for RIPs of the Rep_2 family was built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on 1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/), and sequences showing an identity equal to or higher than 80% were clustered in groups that were highlighted by different background colors. Black dots indicate the RIP of the plasmid used for further comparison in Figs. 5 to 10. doi:10.1128/microbiolspec.PLAS-0039-2014.f13

GENE AND PLASMID FLOW SHAPES THE EVOLUTIONARY ECOLOGY OF FIRMICUTES

As described in previous sections, the acquisition of novel traits encoding adaptive resistance to antimicrobials in *Firmicutes* is mainly due to genes located on plasmids and transposable elements. This acquisition is, certainly, regulated by interactions at genetic and ecological (social) levels. Interplay between genes, mobile genetic elements, and microbial populations and their relation with the host population and local or global environments shapes the plasmid flow. Such flow can be modified by “external” (supra-cellular) changes, including variations in the host population structure and...
size (e.g., mass rearing, crowding) and their associated chemical or behavioral landscape (e.g., use of different antimicrobials, immunization, global food supply, international travel). These changes ultimately determine the density and diversity of particular bacterial populations in particular habitats, leading to ecological specialization, clonalization, and gradual emergence of gene flow barriers (23, 155, 156), a process that mimics the general dynamics of speciation, as bacterial clones and species constitute ecological units of microbial biodiversity.

The challenge to define “units of biodiversity” in microbial community ecology has approached the concept of genes as “defining elements of networks and metacommunities” (155). In such a context, extrachromosomal elements greatly influence the HGT interactions between microbial organisms and are the
building forces for the establishment of “gene exchange communities” (155, 157). The selective power of antimicrobials (antibiotics [Ab], heavy metal, biocides) may then shape this multilevel bacterial population biology (158, 159), involving genes, plasmids (MGEs), bacterial clones and species, and gene exchange communities. The evolutionary tradeoff between early and late stages of adaptation to such selective pressures may determine the local evolvability of clonal and plasmid populations by increasing the number of genotypes resulting from chromosomal and plasmid recombination processes that facilitate further ecological differentiation (18). To establish effective public health interventions to fight the AbR problem in its eco-biological dimension, we then need to define the gene exchange communities relevant for the acquisition, evolution, and spread of resistance (160, 161). Below, we will specifically discuss the role of AbR genes and plasmids in the ecological differentiation of bacterial populations of the main Firmicutes genera.

Antimicrobial Resistance Genes and Bacterial Population Ecology

The environmental origin of AbR genes has been extensively discussed, but very few AbR genes identified in the environment are found in human or animal pathogens, which indicates severe bottlenecks for their acquisition and transmission (162, 163). However, the gut microbiota is increasingly considered a significant reservoir of AbR genes (3), which is supported by studies that associate widely spread AbR genes of relevance in clinical therapy, such as ermB, ermT, ermC (encoding resistance to macrolides), vanB (coding for resistance to glycopeptides), and cfr (coding for resistance to different antimicrobials), with members of the normal microbiota such as species of the Clostridium group XIVa now reclassified as family Lachnospiraceae (Clostridium bolteae, Clostridium innocuum–like, Clostridium lavalense, Clostridium symbiosum) and some lactic bacteria (3, 88, 164–168).

Recent work demonstrates that a given AbR gene (or genetic element such as Tn1549_vanB) may be independently acquired by different clonal populations in the intestine of a particular host (163). Once an AbR gene is present in gut commensals (independent of the origin of the gene), members of the normal intestinal flora of humans and animals can exchange such genes among themselves or with bacterial pathogens, which might be present in low numbers or just be passing through the intestine after being transferred from other body sites or with food intake, using different intermediates in the case of distant bacteria (3, 165, 169).

The rapid emergence in Firmicutes of genes coding for AbR, MerK, and BcK immediately after their introduction and significant (often massive) use in different settings has been demonstrated for chloramphenicol (catA), tetracyclines (tetL), macrolides (ermB), neomycin (aad), gentamicin (aac6aph2), trimethoprim (dfr), beta-lactams (blaZ), and antiseptics (qac) in hospitals during the 1950s to 1970s, and for tylosin (ermC, ermT), phenicols (fex), pleuromutilins (cfr), and zinc-bacitracin in animals during the 1990s, thus supporting the hypothesis of the existence of a previous gastrointestinal reservoir of genes that were selected for the first time as AbR genes (gene exaptation) (84, 88, 91).

Plasmids and Bacterial Population Ecology

The number and types of Firmicutes plasmids and integrative-conjugative elements (currently considered as plasmids under the perspective of evolutionary biology [22]) greatly vary with different bacterial species, certainly as a result of both ecological specialization and selective events resulting from exposure to different anthropogenic activities. Most (if not all) of the contemporary isolates belong to different species of staphylococci, enterococci, lactobacilli, and others contain plasmids of different families in a consistent pattern (for instance, RCR, small theta, or megaplasmids in E. faecium; phenome plasmids in E. faecalis) (7, 68, 71). Such frequent plasmid-bacteria host correspondence indicates a basic coadaptative evolutionary relation between two different types of organisms.

For a long time, plasmids were considered as “organisms,” units of a continuous lineage with an individual evolutionary history, and hence produced evolving populations, in line with the Luria and other seminal works in the field (46, 240). However, plasmids are not necessarily discrete units or individuals as classically considered in evolutionary theory (20, 170, 240). Organisms are units of selection, evolutionary units in a sense “evolutionary individuals,” defined as any entity that, independently from the number of elements that enters into its composition or from its hierarchical level of complexity, is selected and evolves as a unit (170, 171). The frequent out-of-equilibrium events that characterize the interplay between bacterial hosts, plasmids, and gene populations is explained because selective events might act independently on these different evolutionary individuals, as predicted in the “levels of selection” conceptual frame (20, 172–174). However, it is of note that we should recognize “levels of individuality”; for instance, a substantial number of Firmicutes plasmids have a lower-level self-identity than their bacterial hosts (18, 155).
because of the more complex genetic interplay with other mobile genetic elements which in turn are also “leaky individuals,” frequently mosaics of individuals with a partial or contingent self-identity, produced under the effect of adaptive challenges when confronting variable environments (153, 173). Even if this problem of “individual constancy” (176) makes it difficult to study the network of plasmids and hosts in Firmicutes, and such a network were biased by sampling, we should accept the existence of a certain interactive frame.

Valeria Souza, still following Maynard Smith’s ideas about the population structure of bacteria, proposed in 1997 to classify plasmid-bacteria interactions in four patterns, namely, (i) the plasmid-host clonal pattern, where the plasmid phylogeny is mirrored by host phylogeny; (ii) the limited transfer pattern, in which the plasmid flow is limited to closely related (genetically and/or ecologically) strains; (iii) panmictic plasmid spread, in the case of plasmids that circulate among a variety of hosts (the stability of the association being dependent on the benefits and costs of plasmid carriage); and (iv) epidemic plasmid dispersal, in which “successful” plasmids spread in bacterial populations because they provide a clear advantage in high-potency selective landscapes (49, 170). Although illustrative and useful for epidemiological purposes, this single centric view should not replace the complex interplay between different elements that may result in the emergence of different chimeric configurations (49, 177). Therefore, these “patterns” should be currently understood as possible interactive states, even though some of them could be more ephemeral than others, depending on the coevolutionary history, the adaptive demands of the plasmids, and the bacterial populations and communities.

Plasmids and Population Biology of Firmicutes

This section will focus on the genera of Firmicutes that are relevant to the problem of AbR (1).

Streptococcus

The genus *Streptococcus*, a main hub in gene networks in this and other studies (11, 12), is one of the most heterogeneous groups within the phyla *Firmicutes*. Remarkably, the 138 known species of streptococci found as opportunistic pathogens or commensals (many of them zoonotic pathogens) in humans, horses, pigs, cows, and fish have recently been divided into seven species groups on the basis of 16S rDNA gene sequencing, chemotaxonomic approaches, and DNA hybridization, namely the bovis, pyogenic, mitis, mutans, salivarius, anginosus, and unknown groups (178–180). HGT seems to play a relevant role in the adaptation and cohesive-ness of the groups (179). Available information about streptococcal plasmids is scarce, with only a few plasmids being fully sequenced, representing an unbalanced sample of species and ecological groups (Supplementary Table S1). Figure 10 illustrates the 20 AbR plasmids currently found in streptococci.

The streptococcal groups bovis and mutans rarely harbor plasmids, although they can be relevant in the adaptation of particular species. *S. thermophilius*, a non-pathogenic species in the bovis group that is used in the dairy industry (181), contains a set of plasmids harboring heat shock proteins; *Streptococcus mutans*, a member of the human indigenous flora that is transmitted mostly from mother to child, often carries 5- to 6-kb cryptic plasmids that parallel the evolution of lineages associated with racial cohorts and geographical locations (182). Megaplasmids in the group salivarius coding for different lantibiotics favor their persistence in the oral cavity (183). Conversely, the pyogenic group, which is represented by species of clinical interest such as *S. agalactiae* and *S. pyogenes* (also called GAS and GBS, respectively), frequently carry plasmids that code for AbR genes aside from bacteriocins. Inc18 plasmids are widely spread among streptococci and seem to have determined the selection of certain populations resistant to chloramphenicol, aminoglycosides, and macrolides since the late 1970s in different groups of streptococci and enterococci (105, 110, 184). Rep_2 plasmids carrying *erm(T)* seem to have recently spread among GAS and GBS clinical isolates of different countries, having contributed to the increase of macrolide resistance rates in these species since the mid-1990s, either by clonal

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**FIGURE 15** Similarity of *rep*-like sequences encoding RIs of the Rep_3 family. A neighbor-joining tree of gene sequences coding for RIs of the Rep_3 family was built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on 1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2-phylogeny/), and sequences showing an identity equal to or higher than 80% were clustered in groups that were highlighted by different background colors. Black dots indicate the RI of the plasmid used for further comparison in Figs. 5 to 10. Abbreviations: ND, not determined. doi:10.1128/microbiolspec.PLAS-0039-2014.f15
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expansion, in the case of GAS, or by plasmid transfer
ence among unrelated clonal backgrounds, in the case of
GBS (8, 185).

These _erm(T)_-containing plasmids are also spread
among other non-streptococcal species, such as _En-
terococcus, Staphylococcus_, and _Lactobacillus_ (89, 186,
187). Often, streptococcal plasmids are mobilized by
coreisident integrative-conjugative elements belonging to
the ICESa2603 family (188). Resistance to macrolides
(_ermB, mefA_), tetracyclines (_tetM, tetS_, and other mosa
cic _tet_ genes), aminoglycosides (_aph3, aadA6, Tn4001_), or
vancomycin (_vanA, vanB_) is commonly detected among
isolates of this group, but the location of determinants
seems to be linked to transposable elements often in-
volving insertion sequences (reviewed in reference 181).
_Streptococcus suis_, a particularly virulent emerging zoon-
otic pathogen that remains an outlier to the mitis,
sanguinis, and anginosus groups is known to carry
plasmids, although they have been scarcely character-
ized (189, 190). Relevant AbR genes coding for chlor-
amphenicol (_cfr_ _and _fexA_) and lincomamides (_lnu_)
embedded in composite regions similar to those present
in plasmids of _E. faecalis_ have been located in strepto-
coccal plasmids of approximately 100 kb (191). Smaller
plasmids carrying _tetB_ associated with Gram-negative
species have been described (192).

**Enterococcus**

The genus _Enterococcus_ comprises different species,
members of the intestinal flora of animals and humans
able to cause disease in their hosts (193). Although
semenial works in the field of plasmid biology focus on
particular enterococcal plasmids and transposons, such as
pheromone-responsive plasmids or Tn916, which
became paradigms of different mechanisms of conjuga-
tion, the plasmidome of enterococcal species has scarcely
been analyzed (7). Recent studies revealed that most
strains of _E. faecium_ and _E. faecalis_, the two main spe-
cies detected in humans and animals, carry a number
of plasmids of different families that include species-
specific plasmids (e.g., narrow host range RCRs and
RepA_N plasmids such as megaplasmids in _E. faecalis_
and pheromone-responsive plasmids in _E. faecalis_) and
broad host range plasmids (e.g., Inc18), plasmid
chimeras being abundant and still difficult to classify
(Fig. 6 and 7; see previous section and comprehensive
reviews in references 7, 141). Megaplasmids of
_E. faecium_ or pheromone-responsive _E. faecalis_ plas-
mids enhance the ability to colonize, invade, and form
biofilms (65, 126, 154). Conjugative plasmids may in-
fluence the mobilization of nonconjugative elements
and chromosomal regions and facilitate the acquisition
of different adaptive traits and genome evolvability
(71, 125, 126). Most enterococcal plasmids are able
to acquire and disseminate AbR genes by different
mechanisms of genetic exchange. However, the role of
plasmids in the population structure and evolvability of
these enterococcal species has been poorly addressed
(194–197) due to the overrepresentation of recent clin-
cal and animal isolates of specific lineages commonly
associated with AbR included in most studies (7, 141)
and due to the lack of available plasmid sequences.
Similar plasmids have been found in _E. faecium_ and
other enterococcal species that may play equivalent
functional roles in the gastrointestinal tract such as _En-
terococcus avium_, _Enterococcus raffinosus_, _E. durans_,
and _E. hirae_ (195, 198).

AbR genes are located on plasmids that often contain
different replicons associated with different narrow
(RCR, RepA_N) and broad host range (Inc18) plas-
mids. Inc18 streptococcal plasmids greatly influenced
the worldwide increase of aminoglycoside-macrolide
resistance among _E. faecalis_ isolates from humans and
animals during the 1970s (199). They also contributed
to the spread of vancomycin resistance among _E. faec-
iwm_ of animal origin in Europe and _E. faecalis_ from
hospitalized patients in the United States (7, 70, 71).
Diverse narrow host range plasmids have been involved
in local expansions of enterococci conferring resistance
to first-line antibiotics such as gentamicin (Tn4001) or
beta-lactams (ΔTn552 _blaZ_) (152) and beta-lactamase-
producing _E. faecalis_ and _E. faecium_ (152) (200–202),
which highlights the role of endogenous plasmids and
recombination in the adaptation of particular lineages

![FIGURE 16](http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/)

_Similarity of _rep_-like sequences encoding RIPs with the PriCT_–_1 domain._ A
neighbor-joining tree of gene sequences coding for RIPs with PriCT–1 domains was
built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on
1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was
performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/),
and sequences showing an identity equal to or higher than 80% were clustered in groups
that were highlighted by different background colors. Black dots indicate the RIP of the
plasmid used for further comparison in Figs. 5 to 10. Abbreviations: ND, not determined.
(E. faecium ST17, ST18, ST78 and E. faecalis ST6 and ST16) (7, 67, 144, 203).

Analysis of the same AbR genes in different species (cfr, bac, lincomamide resistance genes) reflects the impact of recombination events between genes, MGEs, and different populations of Firmicutes (Staphylococcus, Clostridium, Lactobacillus, Lactococcus, and Enterococcus) and other Gram-negative organisms (200, 204) in the gastrointestinal tract of animals and humans (120, 144, 205, 206).

Staphylococci
These organisms are opportunistic pathogens and members of the commensal flora of skin and mucous membranes of humans and animals (207–209) and, thus, are part of a microbial community with limited contact with members of other main genera of Firmicutes that inhabit distinct body sites (210). Figures 1 and 2 show the limited plasmid connectivity of staphylococci with other genera. However, HGT and the acquisition of AbR and Met\textsuperscript{R} is relevant in the evolvability of this genus, mainly due to genetic exchange events between closely related species (Fig. 3) (9, 211–213). Comprehensive reviews address the essentially clonal population structure of S. aureus (214–216) and other staphylococcal species (207) and also the impact of HGT in the evolutionary history of staphylococcal populations (9, 217–219), with emphasis on the description of the plasmids associated with AbR genetic elements (9, 51, 84, 220) and their influence on the variability of lineages (217, 219, 221–224).

Plasmids, transposons, and staphylococcal chromosomal cassettes (SCC\textit{mec}) are infrequently transferred among isolates of a different origin. A close association of MGE and particular staphylococcal lineages has been suggested (31, 225), with country-specific variations (208, 226). This highlights the relevance of local conditions and the emergence of gene flow barriers in the ecological differentiation of staphylococcal lineages such as in the case of S. aureus CC30 (219, 227). The origin, rapid spread, and evolution of staphylococcal populations resistant to beta-lactams was mainly influenced by the interplay of genetic elements including plasmids (84, 177).

\textbf{Clostridium}
\textit{Clostridium} is a large and extremely heterogeneous genus that has traditionally grouped more than 100 species widely distributed in the gut microbiota of mammals, amphibians, and insects and in soils. An extensive update of clostridial classification is included in the latest edition of Bergey’s Manual, although many unrelated species still retain the \textit{Clostridium} name, causing major confusion in the clostridial taxonomy (228). To date, only 60 plasmids have been fully sequenced, mainly corresponding to \textit{C. perfringens}, \textit{Clostridium botulinum}, and other group I clostridia species (1 \textit{Clostridium butyricum}, 2 \textit{Clostridium kluveyeri}, 3 \textit{Clostridium acetobutylicum}). Some species in which plasmids were analyzed have been moved to other genera such as \textit{Clostridium acidurici} (now \textit{Anaerococcus prevotii} type XII) and \textit{Clostridium thermocellum} (now belonging to the family \textit{Ruminococcaceae}). Several sequenced plasmids correspond to the same strain and are mostly from contemporary isolates, thus limiting the possible knowledge about the role of plasmids in the evolution of these species (Supplementary Table S1). Only narrow host range conjugative plasmids of \textit{C. perfringens} (CpCP) or linear megaplasmids from \textit{C. butyricum} have been associated with AbR.

CpCP plasmids belong to the pCW3 family and are widely spread among isolates of \textit{C. perfringens}, carrying genes encoding AbR (tetracycline [tet\textit{AB(P)}], chloramphenicol [\textit{catP}\textit{Tn4451}], lincomycin [\textit{linuP}\textit{tISCpe8})] and/or enterotoxins, \textit{v-toxin}, or iota-toxin production that determine different toxigenotypes (56, 229–231). All pCW3-like plasmids have a conjugative transfer locus of 11 open reading frames (\textit{orf}s) (\textit{tcp} [transfer \textit{C. perfringens}]) that includes an integrase and a T4CP protein but lacks relaxase (23, 231). A transposable origin similar to that of \textit{Tn916} has been suggested for the \textit{tcp} module of pCW3-like plasmids, which would have acquired a replication machinery specific to this species. Often,

\textbf{FIGURE 17} Similarity of \textit{rep}-like sequences encoding RIPS of the RepA\_\textit{N} family. A neighbor-joining tree of gene sequences coding for replication initiator proteins of the RepA\_\textit{N} family was built using MEGA 6.06. A cut-off equal to or higher than 80% and a bootstrap analysis based on 1,000 permutations were applied to the analysis. Alignment of nucleotide sequences was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2\_phylogeny/), and sequences showing an identity equal to or higher than 80% were clustered in groups that were highlighted by different background colors. Black dots indicate the RIP of the plasmid used for further comparison in Figs. 5 to 10. *Truncated gene. *Similar to \textit{E. faecalis ant6-la} and \textit{aadE}. Abbreviations: ND, not determined. doi:10.1128/microbiolspec.PLAS-0039-2014.f17
C. perfringens isolates harbor more than one pCW3 plasmid, which carry different adaptive traits and partition machineries. The presence of different partition systems explains the coexistence of different plasmids with the same type of RIP in the same cell (56, 231–233).

These plasmids can be transferred (and eventually serve as donors of AbR genes) but cannot replicate in other species such as P. difficile, Clostridium sordelli, or Clostridium septicum, which could explain the confinement of some AbR genes in these populations (234). An evolutionary scenario for CpCP has been reported, with pCW3 (tetAB-P) and pIP401 (tetAB-P and Tn4451) being suggested as the precursors of this family, which would have acquired different toxins by homologous recombination involving composite transposons flanked by insertion sequences (56). Large linear plasmids containing AbR have recently been described in neurotoxigenic C. butyricum, one of the six phylogroups able to produce the botulinum toxin (34, 235). These plasmids contain four beta-lactamase genes, transcriptional regulators and two-component regulatory systems, involved in the regulation of expression of the bont/A gene and a region with a functional CRISPR-cas locus that provides a defense against invading genetic elements present in the intestinal environment.

Acquired resistance to tetracyclines (tetM, tetL, tetK, tetO, tetW), chloramphenicol, macrolides (ermB, lnu), and bacitracin (a bacitracin efflux pump and an over-produced undecaprenol kinase gene located on a genetic island flanked by copies of IS1216) has been reported in human and animal clostridium species including C. perfringens, often associated with conjugative transposons and plasmids widespread in other species (234, 236, 237). A detailed analysis of AbR networks suggests further ecological connections with mobile genetic elements of other prokaryotic groups (Fig. 1 and 2).

**CONCLUSION**

This work offers for the first time an integrated and comprehensive analysis of the dynamics of AbR genes in Gram-positive bacteria and highlights the need for a population view to analyze the problem of antibiotic resistance. The article analyzed the relevance of the plasmidome in the emergence, spread, and maintenance of genes encoding resistance to antimicrobials (antibiotics, heavy metals, and biocides) and their influence on the structure of bacterial populations in the light of evolutionary ecology. A critical revision of plasmid typing systems highlights the limitation of available knowledge about plasmid diversity in this group of bacteria.

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