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Homology among Nearly All Plasmids Infecting Three *Bacillus* Species

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We have surveyed naturally occurring plasmids in strains of *Bacillus subtilis* and the closely related species *B. mojavensis* and *B. licheniformis*. Previous studies have failed to find host-benefiting functions for plasmids of these species, suggesting that these plasmids are nonmutualistic. Only one type of plasmid was found in each plasmid-bearing strain, suggesting that most of the plasmids infecting these *Bacillus* species are in the same incompatibility group. A sample of 18 plasmids from these species ranged in size from 6.9 to 16 kb, with all but 6 plasmids falling into three size groups. These groups differed in the sizes of their host ranges and geographical ranges. All but 1 of the 18 plasmids from these three host species are homologous with one another. The cryptic plasmids from these three species are far less diverse than are plasmids (from other species) that are known to benefit their bacterial hosts. The low-level diversity among these cryptic plasmids is consistent with the hypothesis that host-benefitting adaptations play an important role in fostering the coexistence of plasmid populations, but other explanations for the low-level plasmid diversity are possible. Comparison of the phylogenies of the plasmids with those of their hosts suggests that *Bacillus* plasmids are horizontally transferred in nature at a low rate similar to that found for the colicin plasmids of *Escherichia coli*.

The biodiversity of subcellular organisms, such as plasmids, is poorly understood, as are the forces that determine the diversity of subcellular life. Our understanding of plasmid diversity is limited in part because many of the characteristics typically used to describe the diversity of cellular organisms are not useful for describing plasmids. Morphological characters are of little use, since plasmids frequently have no morphology beyond the structure of their DNA. Some plasmids have easily studied biochemical and physiological characters which could potentially distinguish groups of plasmids (e.g., virulence determinants and resistance to antibiotics and heavy metals) (12). However, plasmids are remarkably modular organisms, and genes for host-benefiting traits are notorious for transferring between otherwise unrelated plasmids (28, 38). Biological and physiological characters are thus not generally useful for establishing the evolutionary relationships among plasmids. One exception is that plasmids may be classified into incompatibility groups, groups of plasmids which cannot be maintained in the same cell because they have the same replication machinery (8, 37). Nevertheless, the genes for replication may be a very small fraction of a plasmid's genome (8, 37). Given the paucity of useful nonmolecular characters, a molecular approach holds the greatest promise for the study of plasmid diversity.

Molecular studies of plasmid genomes have shown a remarkable degree of diversity, even among those plasmids conferring similar phenotypes in the same host. For example, some of the antibiotic resistance plasmids infecting *Staphylococcus aureus* show little homology to one another (i.e., few genes are shared) (32). Little is known, however, about the full diversity of all plasmids infecting a given host species, because almost all studies of plasmid diversity have focused on plasmids that confer a particular phenotype (e.g., antibiotic resistance). Only one species, *Escherichia coli*, has been surveyed for the full diversity of its plasmids, without regard for the phenotypes they might confer (4, 5, 18, 46).

Surveys of *E. coli* plasmids have revealed very high levels of plasmid diversity. Even within single cells there is plasmid diversity, with each strain bearing, on average, four plasmids distinguishable by size (46). Within a single human host, almost all electrophoretically distinguishable *E. coli* strains contained one or more plasmids and strains differed in the sets of plasmids they contained (4). There is a high level of diversity even among plasmids coding for the same class of phenotype; most colicin plasmids show little or no homology with one another (41). The antibiotic resistance plasmids of *E. coli* also fall into multiple incompatibility groups (35).

Although some *E. coli* plasmids confer antibiotic resistance or colicin activity, most *E. coli* plasmids appear to be cryptic, with no known host-benefiting function (5). It is not known whether these plasmids are parasites of their hosts, in that they confer no benefit and grow only at the expense of their hosts, or whether they are mutualists, benefitting their hosts in some manner yet to be determined. In either case, the high frequency of cryptic plasmids, even in a species with many resistance and colicin plasmids, indicates that surveys of plasmid diversity limited to the plasmids of particular phenotypes are likely to underestimate the full diversity of plasmids.

It is not clear whether the high degree of plasmid diversity found in *E. coli* is typical of bacterial species. To address the issue of plasmid diversity more generally, we have investigated the diversity of plasmids in a distantly related group of bacteria, *Bacillus subtilis* and two of its close relatives, *B. mojavensis* (44) and *B. licheniformis*. Our survey of plasmid diversity in *Bacillus* species presents an interesting contrast to *E. coli*. First, *E. coli* is associated with mammalian hosts and *B. subtilis* and its close relatives live in soil (14). Second, almost all plasmids investigated thus far in *B. subtilis* are cryptic. With the exception of two plasmids (16, 47), all plasmids isolated from *B. subtilis* and close relatives fail to show any of the following host-benefiting adaptations: antibiotic resistance, bacteriocin production, and resistance to heavy metals (2, 26, 49, 50, 52).

Plasmids from *B. subtilis* also differ from those of *E. coli* in their conjugative-transfer ability. Many *E. coli* plasmids are large enough to contain a genetic program for auto-transfer (a complex process involving 13 genes and 33.3 kb in F plasmids,

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for example) (54), but almost all *B. subtilis* plasmids appear to be too small to be self-mobilizing (22). With the exception of a few plasmids ranging from 52 to 76 kb, almost all plasmids of *B. subtilis* are smaller than 10 kb (22). While *Bacillus* plasmids would appear to be less mobile than those of *E. coli*, they nevertheless have some potential for horizontal transfer. Some small plasmids of *B. subtilis* can be mobilized by a larger conjugal plasmid (23). In addition, the rolling-circle replication of *B. subtilis* plasmids can yield high-molecular-weight linear concatemers of a plasmid (9), which can be efficiently transferred by transformation (15) or transduction (1, 36).

We have surveyed plasmids found in naturally occurring strains of *B. subtilis*, *B. mojavensis*, and *B. licheniformis* isolated from soils collected in the Gobi Desert, the Sahara Desert, and deserts of the American Southwest. Our survey of *Bacillus* plasmids departs from earlier studies in attempting to quantify the diversity of *Bacillus* plasmids. We have used Southern hybridization to assess to what extent different plasmids have the same sets of genes, and we have used restriction digestes to estimate the percent sequence divergence among plasmids bearing the same set of genes.

We have also addressed whether horizontal-transfer rates for *Bacillus* plasmids might be lower than those for *E. coli* plasmids. The frequency of horizontal transfer was investigated by comparing the phylogenies of plasmids, using restriction digest data, with the phylogenies of their host strains (42). As in previous plasmid surveys (41, 53), our criterion for horizontal transfer was a lack of congruence between the plasmid and host phylogenies.

**RESULTS**

**Comparison of plasmid preparation methods.** A randomly chosen set of 24 strains was screened for plasmids by three small-scale methods. All three methods detected the same plasmids. The remaining 57 strains of this study were screened by one of these methods (that of Lovett and Ambulos [30]). We verified that strains deemed free of plasmids by these small-scale methods were actually free of plasmids by attempting to isolate plasmids from 11 of these strains with the Wizard Miniprep DNA Purification System.

**Survey of strains.** Eighteen of the 81 strains screened were shown to bear plasmids. These strains included 5 of the 50 *B. subtilis* strains, 10 of the 18 *B. mojavensis* strains, and 3 of the 13 *B. licheniformis* strains (Table 1). These species (*n* = 3) were heterogeneous for the relative frequencies of plasmid-bearing strains (*G* = 14.56; *P* < 0.001). For each plasmid-bearing strain, the sum of the lengths of all plasmid DNA restriction fragments for a given enzyme equal the length of the undigested, linear plasmid DNA. We therefore concluded that each plasmid-bearing strain harbors only one type of plasmid.

**Restriction digestion of plasmids.** Restriction analysis of the plasmids showed that the sizes of plasmids varied from 6,900 to 16,000 bp (Table 1), with plasmids falling into the following three size groups: a group of seven plasmids of 8,500 bp (size group 1, including plasmids pBM3, pBM5, pBM13, pBM16, pBM17, pBM18, and pBS4), a group of three plasmids of 7,500 bp (size group 2, including plasmids pBM11, pBM14, and pBM15), and two plasmids of 7,600 bp (size group 3, including plasmids pBL2 and pBL10). Each of the remaining plasmids (pBS12, pBS7, pBS8, pBM9, pBL1, and pBS6) had a unique size and restriction pattern. Plasmids of the same size were very similar in their restriction digests, with generally two or fewer differences in the fragment patterns generated by each enzyme; plasmids of different size classes were generally distinguished by five or more fragment pattern differences (Table 2). Some restriction fragments were shared among size groups and unique plasmids, suggesting that some genes are shared among the plasmids from different size groups (Table 2).

All plasmid-bearing strains of a given size group were found only in a single *Bacillus* species, with one exception. Plasmid pBS4 was found in strain DV8-1.7-4 of the Death Valley group of *B. subtilis* (Fig. 1), while all other strains hosting plasmids of this size group belonged to *B. mojavensis*. Plasmids from size group 1 were found in strains isolated from three continents. In the cases of size groups 2 and 3, all plasmids from each group were isolated from the same site.

**Southern hybridization analysis.** To investigate homologies among plasmids, we performed Southern hybridization analyses of 13 plasmids, including at least one plasmid from each size group and all plasmids with unique sizes (Table 3). For each plasmid analyzed, four of the five plasmids used as probes (pBS4, pBS7, pBS8, and pBS12) yielded identical hybridization patterns. Each of these four probes annealed to all the restriction fragments of 11 plasmids (pBM3, pBM5, pBM13, pBS4, pBM11, pBL2, pBL10, pBS12, pBS7, pBS8, and pBM9), and all of these probes annealed to one of the two restriction fragments of plasmid pBL1 (Table 3). Plasmid pBS6 did not show any homology to any of these four probes. When plasmid pBS6 was used as a probe, it failed to show any homology to any of the other 12 plasmids tested; it annealed only to digests of itself (Table 3).

**The phylogenies of plasmids and their hosts.** We compared the phylogenies of plasmids belonging to size groups 1 and 2 (size group 3 has only two plasmids) with the phylogenies of **MATERIALS AND METHODS**

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**The phylogenies of plasmids and their hosts.** We compared the phylogenies of plasmids belonging to size groups 1 and 2 (size group 3 has only two plasmids) with the phylogenies of
their hosts. The phylogeny of host strains and the estimates of their sequence divergence were based on the restriction digest of PCR-amplified segments of three genes (gyrA, rpoB, and polC) (42). The phylogeny of plasmids belonging to each size group was based on the presence and absence of restriction fragments from the 12 restriction endonucleases, using Swoford’s PAUP 3.1 (48) parsimony analysis. Each plasmid phylogeny was rooted by using the plasmids from the other size group as an outgroup.

The plasmid and host phylogenies for size group 1 were largely congruent, suggesting that the transmission of plasmids has been largely vertical (Fig. 2). The one exception to complete congruence was that plasmid pBM13 was an out-group with respect to the other plasmids in its size group, while its host strain, TU-A-10, was not. Our best estimate for the number of horizontal-transfer events in this phylogeny is therefore one. However, the difference between the plasmid and host phylogenies in the placement of pBM13 and its host is only weakly supported by bootstrap analysis. Of bootstrap replicates, 89% find pBM13 to be the out-group among these plasmids, but only 56% of bootstrap replicates support the placement of a strain other than pBM13’s host as the out-group in the strain phylogeny. Clearly, inferring the number of transfer events from these data must be viewed with reservations, as the bootstrap values do not provide confidence in the phylogenies. However, it is this type of approach (with more strains and more restriction enzymes) that will eventually allow us to estimate more precisely the horizontal-transfer rates of plasmids in nature.

Comparison of the phylogeny of the three plasmids of size...
in the frequencies of strains bearing plasmids, with plasmid-bearing strains making up 56% of B. mojavensis strains, 23% of B. licheniformis strains, and 10% of B. subtilis strains. Previous studies have reported the frequency of plasmid-bearing B. subtilis strains at higher levels, from 10 to 67% (average, 34%) (26, 31, 49, 50, 52). However, our data do not necessarily conflict with these studies. Because B. mojavensis strains were until recently misdiagnosed as B. subtilis strains (44), it is possible that the fraction of plasmid-bearing B. subtilis strains was previously overestimated by including B. mojavensis strains.

**Lack of diversity among Bacillus plasmids.** We assayed the relatedness among the plasmids of these three Bacillus species by restriction digest analysis and Southern hybridization. Southern hybridization data suggested that all but one of the plasmids from these three species are homologous with one another over most or perhaps all of their genomes. It is unlikely, however, that all plasmid pairs have exactly the same set of genes, since the plasmids vary in size from 6,900 to 9,800 bp.

To investigate the degree of homology within each size group (i.e., the fraction of the genome that is homologous), we used restriction digest analysis. The similarity of restriction digest bands within each group was consistent, with nucleotide sequence divergence of 5% or less for whole plasmids (Tables 4 and 5). This degree of sequence similarity places an upper boundary on the regions of the plasmid genome that can be nonhomologous. (That is, genes that are not shared across plasmids could account for no more than ~5% of the plasmid genome, or ≤425 bp.) This upper boundary would apply if none of the restriction digest divergence were due to nucleotide sequence substitution within homologous regions. We conclude that plasmids of the same size in our study represent clusters of plasmids with very high levels of homology.

Restriction digest analysis cannot address the degree of homology between size groups. The small fraction of bands shared between size groups is consistent with sequence divergence levels of greater than 10%, and analyses based solely on the sharing of restriction fragments can be grossly inaccurate at such high levels of divergence (33).

This is the first instance, to our knowledge, in which three host species are known to be infected almost exclusively by one set of homologous plasmids. The lack of plasmid diversity in Bacillus species stands in sharp contrast to the case of E. coli (8, 40, 41).

**Possible differences in host range.** The three plasmid size groups differed in the breadth of hosts in which they were borne. Plasmids from size group 1 were found in strains covering the entire phylogenetic range of B. mojavensis as well as in one strain of B. subtilis. The DNA sequence divergence between the most distant of these host strains was 10.1%. The geographic range of these plasmids was also very broad, with strains from three continents harboring group 1 plasmids. In contrast, the three plasmids of size group 2 were found only in the Gobi Desert strains and only among very closely related strains of B. mojavensis (with ≤0.2% sequence divergence between strains). It will be interesting to see if the more limited range of group 2 holds up under more extensive sampling. This would indicate that compared with group 1 plasmids, group 2 plasmids infect a narrower set of hosts or confer a larger cost on strains from diverse lineages.

**One plasmid per host.** All of the plasmid-bearing strains investigated in this study harbor only one plasmid. This result is consistent with previous surveys of B. subtilis plasmids (2, 26, 31, 50, 52; for conflicting results, see reference 49). In contrast, multiple infection is quite common for the plasmids of E. coli, most of whose strains have two or more plasmids (4). One explanation for the lack of multiple infections in Bacillus spe-
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Length(s) of restriction fragment(s) (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBM5</td>
<td>2,200, 1,800, 1,300, 1,100, 750, 550, 450</td>
</tr>
<tr>
<td>pBM17</td>
<td>2,200, 1,800, 1,300, 1,100, 750, 550, 450</td>
</tr>
<tr>
<td>pBM18</td>
<td>2,200, 1,800, 1,300, 1,100, 750, 550, 450</td>
</tr>
<tr>
<td>pBM16</td>
<td>2,200, 1,800, 1,300, 1,100, 750, 550, 450</td>
</tr>
<tr>
<td>pBS4</td>
<td>2,450, 1,800, 1,300, 1,250, 750, 550, 450</td>
</tr>
<tr>
<td>pBM13</td>
<td>3,100, 2,200, 1,300, 750, 550, 450</td>
</tr>
<tr>
<td>pBM11</td>
<td>2,800, 1,900, 1,400, 1,400</td>
</tr>
<tr>
<td>pBM15</td>
<td>2,800, 1,900, 1,400, 1,400</td>
</tr>
<tr>
<td>pBM14</td>
<td>2,800, 1,900, 1,400, 1,400</td>
</tr>
<tr>
<td>pBL2</td>
<td>7,600, 7,600, 4,900, 1,700, 1,000</td>
</tr>
<tr>
<td>pBL10</td>
<td>7,600, 7,600, 6,600, 1,000</td>
</tr>
<tr>
<td>pBS12</td>
<td>3,300, 1,900, 1,400, 1,400, 800</td>
</tr>
<tr>
<td>pBS7</td>
<td>3,800, 2,500, 550</td>
</tr>
<tr>
<td>pBS8</td>
<td>4,100, 2,400, 1,700</td>
</tr>
<tr>
<td>pBM9</td>
<td>2,600, 2,000, 1,100, 1,000, 800, 650, 550</td>
</tr>
<tr>
<td>pBL1</td>
<td>4,500, 2,700</td>
</tr>
<tr>
<td>pBS6</td>
<td>4,800, 3,600, 2,400, 2,400</td>
</tr>
</tbody>
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- MOD, restriction enzyme did not digest the plasmid because of a lack of recognition sites.
- a, MOD, restriction enzyme did not digest the plasmid because it was modified against the restriction enzyme.
cies is that all or most of the Bacillus plasmids may belong to the same incompatibility group (35), so multiple-plasmid genotypes cannot be stably inherited in the same cell line. This possibility is supported by the result that all but one of the plasmids in our study appear to be homologous; if they all have the same origin of replication, they should be in the same incompatibility group. If this explanation is correct, then the lack of plasmid diversity at the host-species level is the cause of the low-level diversity at the level of single cells. This interpretation is supported by the fact that all of the B. mojavensis plasmids from groups 1 and 2 have the same replication protein (coded by rep) (6, 17).

Another explanation for finding one plasmid per strain is that each plasmid may have a very limited host range that does not overlap with those of other plasmids. This mechanism may be responsible for preventing the coinfection of plasmids of size group 2 with some plasmids of unique size, since these plasmids appear to have limited ranges. However, the limited host range hypothesis is unlikely to explain the nonoverlapping plasmids of size group 1 among themselves or with other plasmids, since plasmids of size group 1 are distributed widely both phylogenetically and geographically.

**Comparison of sequence diversity within populations of plasmids and their hosts.** For plasmid size groups 1 and 2, the nucleotide sequence diversity among the plasmids of each group is similar to that among the host chromosomes for chromosomal genes gyrA, polC, and rpoB. Two of these chromosomal genes are highly conserved, with only 2% amino acid sequence divergence at rpoB (25, 29) and 4% divergence at polC between E. coli and Salmonella typhimurium (24, 51); the third gene, gyrA, shows a more typical level of conservation, with 11% amino acid sequence divergence between E. coli and Klebsiella pneumoniae (11, 55) (sequence divergence for all three genes was determined by using Megalign software [DNASTAR, Inc.] for sequence alignment). These results suggest a high level of constraint on plasmid sequence evolution.

**Infrequent horizontal transfer.** To investigate the prevalence of horizontal plasmid transfer, we compared the phylogenies of plasmids and their hosts for size groups 1 and 2. The host strain phylogeny, based on restriction digests of genes gyrA, polC, and rpoB, is likely to be an accurate estimate of the true strain phylogeny, since these three genes are unlinked with respect to bacterial recombination (39), and the rate of recombination of chromosomal genes in Bacillus species is extremely low, similar to the rate of neutral mutations (42; for conflicting interpretations, see references 20 and 21). Our best estimate of the number of horizontal-transfer events for each phylogeny is one, although more strains and more restriction data would be required for confidence in this estimate.

Possible evidence for an additional horizontal-transfer event comes from the observation that the plasmids of size group 1 were found entirely within B. mojavensis strains, except for one plasmid found in a strain from the Death Valley group of B. subtilis strains (plasmid pBS4 in strain DV8-1.7-4). This result is consistent with one of the following explanations: (i) the plasmid of DV8-1.7-4 was transferred horizontally from B. mojavensis into this B. subtilis strain or (ii) the common ancestor of B. subtilis DV8-1.7-4 and B. mojavensis contained a plasmid of size group 1 which was lost independently in many descendant lineages.

The finding that host and plasmid phylogenies differ infrequently as we have observed suggests that the horizontal transmission of plasmids is rare in these Bacillus species. This result is consistent with the observation that Bacillus plasmids are too small to code for their own transfer. This result suggests that alternative modes of Bacillus plasmid transfer, such as transformation (15) and transduction (1, 36), occur rarely at most in nature.

The colicin E1 plasmids of E. coli have shown a pattern of horizontal transfer very similar to that of Bacillus plasmids. A comparison of the phylogeny of eight colicin plasmids with the phylogeny of their hosts indicated one plasmid transfer event (41). This result is almost identical to that found with size group 1 Bacillus plasmids, for which one plasmid transfer event was detected in the phylogenies of seven plasmids and hosts.

![Figure 2. Comparison of the phylogeny of plasmids from size group 1 with the phylogeny of their host strains.](Image)

![Figure 3. Comparison of the phylogeny of plasmids from size group 2 with the phylogeny of their host strains.](Image)
plasmids infecting this species (10). Plasmids are more diverse than their host, but even with the cryptic plasmids of E. coli as well as with plasmids of other species. Alternatively, one may argue that the low-level diversity of Bacillus plasmids is due to their low-level frequency of horizontal transfer. Low-level diversity might be expected because the equilibrium frequency of any plasmid is determined in part by its rate of horizontal transfer (27), and our phylogenetic analysis has indicated very low levels of horizontal-transfer frequency for Bacillus plasmids. However, we argue that the rarity of horizontal transfer cannot fully explain the low-level diversity of Bacillus plasmids. The colicin E1 plasmids of E. coli have shown an incidence of horizontal transfer similar to that of the Bacillus plasmids, yet the colicin plasmids are much more diverse.

In summary, the extremely low levels of diversity among Bacillus plasmids are consistent with the hypothesis that the parasitic nature of these plasmids depresses their diversity.

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