Host Function Specified by *Bacillus pumilus* Plasmid pPL7065

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Plasmid pPL7065 ($\sim 4.7 \times 10^6$ daltons; ~ 20 copies per chromosome) determines the production of, and immunity or resistance to, a killing activity in strains of *Bacillus pumilus*. Plasmid pPL7065 is compatible with plasmid pPL576 ($\sim 28 \times 10^6$ daltons; ~ 2 copies per chromosome).

Recent efforts to demonstrate naturally occurring plasmid systems in the related species *Bacillus subtilis* and *Bacillus pumilus* have relied primarily on the identification of strains that harbor covalently closed circular duplex (CCC) deoxyribonucleic acid (DNA) molecules of homogeneous size, buoyant density, and of limited copy number (7, 8, 9, 14). Presently only two *Bacillus* plasmids have been associated with, or shown to specify, host characteristics. *Bacillus* plasmid pPL576 ($\sim 28 \times 10^6$ daltons; $\rho = 1.698$; ~ 2 copies per chromosome) is present in the oligosporogenic *B. pumilus* strain NRS576 (4). Variants of this strain that form spores at high frequency, the W mutants, lack pPL576 (4, 6). Accordingly, the presence of pPL576 correlates with the reduced ability of the host to form spores. More recently the plasmid pPL10 ($\sim 4.4 \times 10^6$ daltons; $\rho = 1.698$; ~ 20 copies per chromosome) was shown to determine the production of, and immunity or resistance to, a killing activity in strains of *B. pumilus* (10). The positive host phenotype determined by pPL10 permitted initiation plasmid transduction and transformation studies in *Bacillus* (1, 10). In the present investigation we have reexamined a *B. pumilus* plasmid, pPL7065, previously identified as a cryptic plasmid (9). Evidence is presented that demonstrates that pPL7065 specifies a killing activity in host strains.

B. pumilus ATCC 7065 was previously shown to harbor about 20 copies per chromosome of a small plasmid that sedimented at 29S, indicating an approximate molecular weight of 6×10^6 (9). We now find that the CCC DNA molecules present in this strain consistently sediment at $26 \pm 1S$, indicating a molecular weight of approximately 4.7×10^6 (2). The basis for this discrepancy is not clear, although we feel it unlikely that either measurement was incor-

rect. It is possible that the original subline of ATCC 7065 studied (since discarded) harbored a somewhat amplified form of pPL7065, although this is not known.

Strain ATCC 7065 exhibited a weak killing activity against *B. pumilus* strains NRS576 and W20 when the strains were cross-streaked on tryptose-blood agar base (Difco) and incubated at 37°C for 18 h. Attempts to isolate non-killing derivatives of ATCC 7065 by treatment of cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or by growth of ATCC 7065 in Penassay broth (Difco) containing 10^{-5} M ethidium bromide or 30 μ g of acridine orange per ml were unsuccessful. The transducing bacteriophage PBP1 (3, 5) was therefore grown on ATCC 7065 and used to generate Kill⁺ transductants of W20 and NRS576 in the manner previously described (1, 10). Kill⁺ transductants of both recipients occurred at a frequency of about 1 transductant per 10^8 plaque-forming units. Each of five cloned Kill⁺ transductants of W20 retained the nutritional requirement of this strain (adenine requiring). Lysates prepared from late-log phase cells of each Kill⁺ transductant, labeled with [³H]thymidine (New England Nuclear) as previously described, were centrifuged in cesium chloride-ethidium bromide gradients for 36 to 40 h (10). Each harbored $3.7 \pm 0.5\%$ of the total cell DNA as CCC molecules. The CCC DNA from each Kill⁺ transductant sedimented at $26 \pm 1S$ relative to [¹⁴C]thymidine-labeled T7 DNA reference, which was assigned an S value of 32 (13).

B. pumilus strain NRS576 harbors about two copies per chromosome of the 28×10^6 dalton plasmid pPL576 (4). Each of two Kill⁺ transductants of NRS576 harbored $7.5 \pm 0.4\%$ of the total cell DNA as CCC DNA molecules. Sedimentation of this CCC DNA through 5 to 20% neutral sucrose gradients demonstrated the resident plasmid, pPL576, and a 26S species corresponding to the CCC form of pPL7065. The ratio of pPL7065 molecules to pPL576 molecules

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was estimated as 15:1, determined by weighing areas under the corresponding peaks in sucrose gradients. Accordingly, pPL7065 and pPL576 are compatible plasmids.

B. pumilus W20 harboring the 4.4×10^6 dalton plasmid pPL10, which determines a Kill⁺ host characteristic (10), and strain W20 harboring pPL7065 exhibited cross-killing activity when the two were cross-streaked. Therefore, the presence of one of the plasmids did not confer immunity to the killing activity determined by the other. Efforts to construct derivatives of W20 by transduction that harbored both plasmids have been unsuccessful, suggesting that the two plasmids may be incompatible.

Plasmids pPL10 and pPL7065 are not identical. The two plasmids can be distinguished by agarose gel electrophoresis (Fig 1). Plasmid pPL10 (4.4×10^6 daltons) contains a single *Hind*III-sensitive site (10). Agarose gel electrophoresis of a *Hind*III-digested mixture of differentially labeled pPL10 and pPL7065 demonstrated that *Hind*III generates at least two fragments from pPL7065, but neither of these fragments is comparable in size to the *Hind*III-generated linear form of pPL10 (Fig 1).

Plasmid pPL7065 has been transferred by PBP1 transduction to three additional strains of *B. pumilus*: NRRL B-3275, L9S1, and NCIB 8600 (Table 1 [3]). In each case at least three Kill⁺ transductants of each strains were shown to maintain CCC DNA that sedimented at $26(\pm 1)$ S. We previously reported that PBS1 was an inefficient vector for transduction of plasmid pPL10 (1). Similarly, PBS1 transduction of pPL7065 was extremely reduced relative to PBP1 transduction of the plasmid (Table 2).

The killing activity determined by pPL7065

TABLE 1. Strains of *Bacillus pumilus*

Strain designation	Relevant properties	Reference/source
NRS 576	pPL576 ⁺	4
W20	pPL576 ⁻ ; Ade ⁻	4
W40	pPL576 ⁻ ; Ade ⁻ Ser ⁻ Cys ⁻	1
ATCC 7065	pPL7065 ⁺	9
NRRL B-3275	Wild type	11
ATCC 12140	pMB1 ⁺ pMB2 ⁺	10
L9S1	pMB1 ⁺ pMB2 ⁻ Lys ⁻	10; Plasmid-negative derivative of ATCC 12140
NCIB 8600	Wild type	11

FIG. 1. Agarose gel electrophoresis of mixtures of pPL7065 and pPL10 intact and *Hind*III digested. Approximately 0.1 μ g of pPL7065 labeled with [³H]thymidine (●) was mixed with 0.1 μ g of pPL10 labeled with [¹⁴C]thymidine (○) in 100 μ l of 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.2. Half the mixture was digested with *Hind*III endonuclease as previously described (10) and held at 65°C for 10 min. The undigested (A) and the *Hind*III-digested (B) portions were mixed with an equal volume of 0.2% bromophenol blue and 20% sucrose in TES buffer (N-tris(hydroxymethyl)methyl-2-aminomethane-sulfonic acid; 10). A 50- μ l sample of each was applied to 0.7% agarose gels as previously described (10). Electrophoresis was at room temperature for 18 h at 34 V/gel. Gels were then sliced into 2-mm sections. Each slice was dissolved in concentrated ammonium hydroxide and counted in Hydromix (Yorktown Research). Migration proceeded from left to right.

TABLE 2. Transduction of plasmid pPL7065 by bacteriophages PBP1 and PBS1^a

Transducing phage	Infectious ^b titer/ml	Transductants per ml		Transductants per PFU	
		Cys ⁺	Plasmid ⁺	Cys ⁺	Plasmid ⁺
PBP1	5 × 10 ¹⁰	465	1 × 10 ⁵	9 × 10 ⁻⁹	2 × 10 ⁻⁶
PBS1	1 × 10 ⁹	1 × 10 ⁴	80	1 × 10 ⁻⁵	8 × 10 ⁻⁸

^a Phage were propagated on W20 (pPL7065); recipient was W40 (Ade⁻ Cys⁻ Ser⁻). PFU, Plaque-forming units.

^b Indicator cells for plaque assay were W40.

was not active against *B. subtilis* 168, as judged by the lack of detectable antagonism when strain W20 (pPL7065) was cross-streaked with JH86 (a spore-negative derivative of *B. subtilis* with the genotype *spoB phe-1 trpC2* [10]). Efforts to transform pPL7065 into JH86 by the technique of congression as previously described (10) were unsuccessful.

The occurrence of plasmid-determined killing activities is well established among gram-negative bacteria. In those cases where the killing property has been studied in detail, the activity has been shown to be determined by a protein (bacteriocin or colicin [12]). The chemical nature of the killing activities determined by pPL10 and pPL7065 are unknown at present.

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