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The Biopesticide *Paenibacillus popilliae* Has a Vancomycin Resistance Gene Cluster Homologous to the Enterococcal VanA Vancomycin Resistance Gene Cluster

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We have previously identified, in *Paenibacillus popilliae*, a 708-bp sequence which has homology to the sequence of the enterococcal *vanA* gene. We have performed further studies revealing five genes encoding homologues of VanY, VanZ, VanH, VanA, and VanX in *P. popilliae*. The predicted amino acid sequences are similar to those in VanA vancomycin-resistant enterococci: 61% identity for VanY, 21% for VanZ, 74% for VanH, 77% for VanA, and 79% for VanX. The genes in *P. popilliae* may have been a precursor to or have had ancestral genes in common with vancomycin resistance genes in enterococci. The use of *P. popilliae* biopesticidal preparations in agricultural practice may have an impact on bacterial resistance in human pathogens.

In the United States, the percentage of nosocomial enterococcal infections caused by vancomycin-resistant enterococci (VRE) is increasing. This increase poses important problems, including the dearth of available antimicrobial therapy for these organisms and the possibility that vancomycin resistance genes can be transferred to other gram-positive bacteria, especially *Staphylococcus aureus*. Five glycopeptide resistance types in enterococci—VanA, VanB, VanC, VanD, and VanE—have been described and can be distinguished on the basis of the level and inducibility of resistance to vancomycin and teicoplanin, transferability of glycopeptide resistance, and presence of specific ligase genes. VanA and VanB types of glycopeptide resistance have been associated with outbreaks of VRE infections and are readily transferred from enterococci to other gram-positive organisms. One of the most worrisome examples of this is the transfer of high-level vancomycin resistance from enterococci to *S. aureus* in the laboratory (13). VanA vancomycin resistance has also been transferred in vitro by conjugation or transformation to *Streptococcus sanguis*, *Lactococcus lactis*, *Streptococcus pyogenes*, and *Listeria monocytogenes* (4, 16). In addition to laboratory experiments, the *vanA* gene has been found in vancomycin-resistant clinical isolates of *Cellulomonas turbata*, *Arcanobacterium haemolyticum*, and *Bacillus circulans* (8, 16) and the *vanB* gene has been found in a vancomycin-resistant isolate of *Streptococcus bovis* (17). Reduced susceptibility of *S. aureus* to vancomycin has recently been described in Japan and in the United States, although the mechanism of resistance to vancomycin in these isolates is distinct from that in VRE (19).

VanA-type glycopeptide resistance is characterized by acquired inducible resistance to both vancomycin and teicoplanin. It is mediated by Tn*1546* or closely related elements which encode nine polypeptides assigned to groups with different functions: transposition functions; regulation of vancomycin resistance genes (VanR and VanS); synthesis of depsipeptide D-alanyl–D-lactate, which when incorporated into the pentapeptide peptidoglycan precursor forms a precursor to which vancomycin and teicoplanin bind with reduced affinity (VanH and VanA); and hydrolysis of precursors of normal peptidoglycan (VanX and VanY). The function of VanZ is unknown (2). The *vanB* and *vanD* gene clusters have homology to the *vanA* gene cluster but have been less well studied (5, 6, 14).

Vancomycin resistance present in nonenterococcal organisms may have been transferred to enterococci under the pressure of increased oral and parenteral vancomycin use in clinical practice and the use of glycopeptides (avoparcin and orienticin) in animal husbandry (3, 22). The source of these vancomycin resistance genes is unknown. It has recently been hypothesized that the source may be glycopeptide-producing organisms (11). Other environmental organisms may have been the more direct source. *Paenibacillus* (formerly *Bacillus*) *popilliae*, a vancomycin-resistant biopesticide (vancomycin MIC, 800 μg/ml; teicoplanin MIC, <1 μg/ml) (18), has been used in the United States for more than 50 years for suppression of Japanese beetle populations; *P. popilliae* causes milky disease of Japanese beetle larvae. We have previously identified, in *P. popilliae*, a 708-bp fragment which has homology to a portion of the enterococcal *vanA* gene (18). The putative ligase gene in *P. popilliae* has 77% nucleotide identity to the sequence of the *vanA* gene and was designated *vanE* (18). Since our original description, another *vanE* gene has been described (7); therefore, we have renamed the putative ligase gene in *P. popilliae vanF*. The purpose of this study was to determine whether *vanY*, *vanZ*, and *vanX*- and *vanH*-like genes are present in *P. popilliae*.

MATERIALS AND METHODS

P. popilliae ATCC 14706 was studied. DNA was extracted by using DNA-STAT (Tel-Test, Inc., Friendswood, Tex.). PCR amplification was performed as previously described (15). The PCR primers used included published primers designed to amplify the enterococcal *vanH* gene (12) and the *P. popilliae vanF* gene (18), newly designed primers designed (based on the published sequence of *vanX*) to amplify fragments of the *P. popilliae* $vanX_F$ gene (described herein), and newly designed primers based on sequences derived from restriction site PCR (Table 1). Restriction site PCR was used to extend the sequence in the 5′ and 3′ directions; restriction site PCR involves PCR using four separate universal primers which are representative of given restriction enzyme sites (restriction site

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TABLE 1. PCR primers used in this study

Forward primer	Forward primer		Reverse primer	Reverse primer		Product size (bp)
	Sequence	Location on sequence shown in Fig. 1		Sequence	Location on sequence shown in Fig. 1	
BPOP-50F	TTTAATATTTACCCCACA	Upstream	BPOP-159R	TACTAACGCCATCCTCTT	415	458
BPOP-46F	AAGGGAATCTACTCTTGG	221	BPOP-158R	TTCAGGCGCTTTATCCAT	576	356
BPOP-40F	GACTTGGATGAGCAAAGCAG	442	BPOP-1308R	AGCGCAAGGTAGAACAGAAA	1127	686
BPOP-walk5′F	AATTCAATATGAACCTTGGCATATT	654	BPOP-4R	CACTAGCGCAAGGTAGATCA	1131	478
BPOP-1086F	TTGATGGAGTAATTGTGACC	887	BPOP-1909R	GGCAGAGCTTGTAATGG	1726	840
BPOP-298F	AAAGGTAAAGTTAGGAGACG	951	BPOP-147R	CCTAACGGCATAAACAGAAT	1274	323
BPOP4-F	TGATCTACCTTGCGCTAGTGT	1112	BPOP-433R	CATTGCGTTGGTTTCCGATA	1747	636
vanHEf	TCGCCACGCTTTGGTGTCATAC	1685	BPOPREV	AATTGCTTTCGCCGTCTC	3416	1,732
vanE-FOR	CTCGGCTGTAAAGGTCTT	3426	vanX-REV	AAAATAGSTATYGGGGTATGG	4208	783
BPOPX-2356F	GAGCGGCTTCGATTTTATGGA	4030	BPOPX-2739R	CCATGCTGGAACACGATGAGGT	4434	405
BPOP-3039F	AACGCCGTCGGAGTCCATTT	4334	BPOP-3670R	TATAGTTGCCGAATTTGTTTC	4800	467
BPOP-3508F	GGCGAAGAGGCTTAATATGC	4616	BPOP-walk3′R	AGGCACATGGCTCGTTGATTTAAT	5025	410
BPOP-4311F	TTGCTTCTATCGTTTTGTTA	4965	BPOP-5754R	CAGCTACTGATGAATTAGTG	5545	581
BPOP-5667F	TGAATGGTATCGCCGCAGTA	5468	BPOP-5970R	TCAGGTGAAATACCATAT	5768	301
BPOP-5251F	CCGCACAGCAACTATTTA	5652	BPOP-6010R	TCTGCACCTGCCATCAA	Downstream	548

primers) and a specific (first-stage) primer from one end of the known sequence (21). This is followed by nested PCR with the restriction site primers and an internal specific (second-stage) primer after which the product is sequenced by using a third internal specific primer (21). All sequences were confirmed in both the 5′-to-3′ and the 3′-to-5′ directions.

For sequencing, 6 μl of the PCR mix, 1 μl of a 1-U/μl concentration of shrimp alkaline phosphatase, and 1 μl of a 10-U/μl concentration of exonuclease I (United States Biochemical) were incubated at 37°C for 30 min followed by 80°C for 15 min. One microliter of dimethyl sulfoxide and 1 μl of a 3.2-μM sequencing primer were then added. The DNA sequence was determined in both the 5′-to-3′ and the 3′-to-5′ directions with a *Taq* dideoxy terminator cycle sequencing kit and a 373A DNA sequencer (Applied Biosystems, Foster City, Calif.) by using a series of internal sequencing primers that provided appropriate coverage of the *van* genes. The sequence data were analyzed with Sequencher 3.0 (Gene Codes Corp., Ann Arbor, Mich.).

Nucleotide sequence accession number. The nucleotide sequence of the gene cluster of $vanY_F$, $vanZ_F$, $vanH_F$, *vanF*, and $vanX_F$ of *P. popilliae* ATCC 14706 has been submitted to GenBank and given accession no. AF155139.

RESULTS

A total of 6,177 bp of *P. popilliae* DNA, encompassing *vanY*, *vanZ*, *vanH*, *vanA*, and *vanX* enterococcal gene homologues, was sequenced (Fig. 1). VanY and the putative *P. popilliae* VanY protein, $VanY_F$, have 61% predicted amino acid identity (Fig. 2), although the *vanY* gene is 30 bp longer than the $vanY_F$ gene. $VanY_B$ and $VanY_F$ have 25% predicted amino acid identity, although $vanY_F$ is 72 bp longer than $vanY_B$. VanZ and the putative *P. popilliae* VanZ protein, $VanZ_F$, in contrast, have only 21% predicted amino acid identity (Fig. 2), and the $vanZ_F$ gene is 135 bp longer than the *vanZ* gene. *vanH* and the *P. popilliae vanH* gene homologue, $vanH_F$, have 75% nucleotide identity and 74% predicted amino acid identity (Fig. 2). $vanH_B$ and $vanH_F$ have 69% nucleotide identity and 65% predicted amino acid identity, although $vanH_B$ has 3 more bp at the 5′ end than $vanH_F$ and *vanH* do. $VanH_D$ and $VanH_F$ have 60% predicted amino acid identity. *vanA* and *vanF* (the *P. popilliae vanA* gene homologue) have 77% nucleotide and predicted amino acid identity. *vanB* and *vanF* have 69% nucleotide identity and 67% predicted amino acid identity, although *vanB* has a 3-bp deletion, as compared to *vanF* (and *vanA*). VanD and VanF have 64% predicted amino acid identity. *vanX* and the *P. popilliae vanX* gene homologue, $vanX_F$, have 80% nucleotide identity and 79% predicted amino acid identity, although $vanX_F$ has 63 additional bp at the 3′ end when compared to *vanX*. $vanX_B$ and $vanX_F$ have 74% nucleotide identity and 73% predicted amino acid identity, although $vanX_F$ has an additional 63 bp at the 3′ end when compared to $vanX_B$. $VanX_D$ and $VanX_F$ have 69% predicted amino acid identity, although $vanX_F$ has 63 additional bp at the 3′ end when compared to *vanX*. The orientations of the $vanH_F$, *vanF*, and $vanX_F$ genes are identical to the orientations found in VRE (Fig. 2). Downstream of this gene cluster is an open reading frame encoding a putative protein of unknown function which has 75% amino acid identity to the putative oxidoreductase in the *inlA* 5′ region in *Listeria monocytogenes* (9).

DISCUSSION

We have detected a vancomycin resistance gene cluster in *P. popilliae* which is homologous to the vancomycin resistance gene cluster in VanA VRE. In addition to the considerable similarity in amino acid composition noted, the orientation and alignment of the $vanH_F$, *vanF*, and $vanX_F$ genes are identical to the orientation and alignment of homologous genes in VanA VRE (except that $vanX_F$ has an additional 63 bp at the 3′ end) (Fig. 2). The 3′ extension of $vanX_F$ is not present in the genes encoding VanX and its homologues in VanA and VanB enterococci nor is it present in *Streptomyces toyocaensis* or *Amycolotopsis orientalis* (11). The signature overlap of the 5′ end of

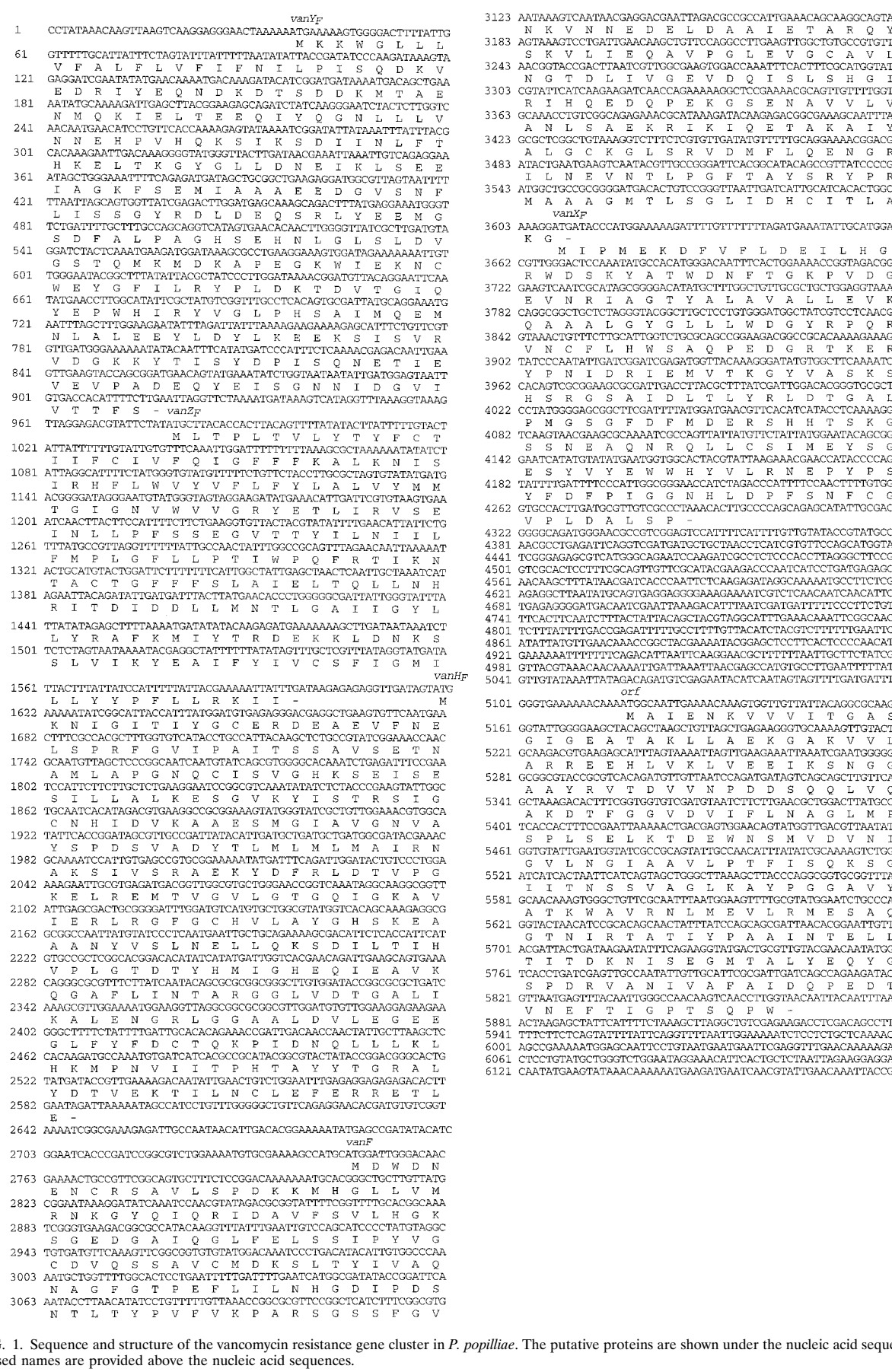

FIG. 1. Sequence and structure of the vancomycin resistance gene cluster in *P. popilliae*. The putative proteins are shown under the nucleic acid sequence, and the proposed names are provided above the nucleic acid sequences.

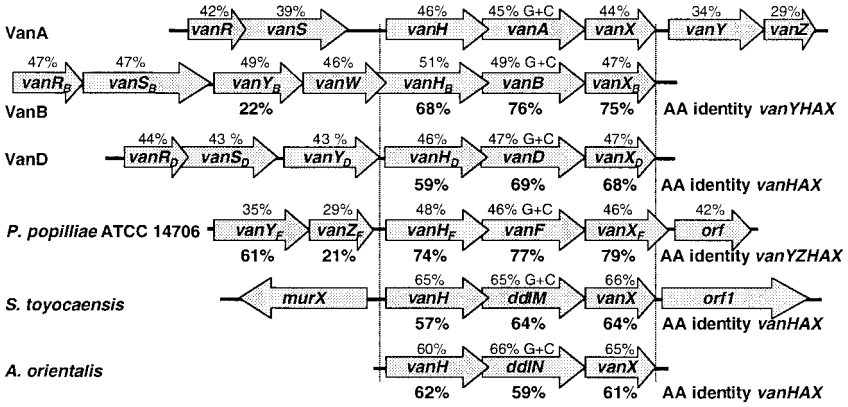

FIG. 2. Alignment of glycopeptide resistance gene clusters of VanA, VanB, and VanD VRE, *P. popilliae* (as described herein), and the glycopeptide-producing organisms *Amycolotopsis orientalis* and *Streptomyces toyocaensis* (1, 6, 7, 12, 15). The percent amino acid identity to *vanY*, *vanZ*, *vanH*, *vanA*, and *vanX* products are shown below the respective genes (below the arrows). The percent G+C content of each gene is shown above the genes (above the arrows).

vanF with the 3′ end of *vanH*, as described by Marshall et al., has been identified (11). That the spatial arrangements of the $vanH_F$, *vanF*, and $vanX_F$ genes are maintained suggests a common ancestry with vancomycin resistance genes in VanA and VanB VRE. *vanY* and *vanZ* gene homologues were also found. There are several implications of these findings. That *P. popilliae* possesses this gene cluster implies that *P. popilliae* has a glycopeptide resistance mechanism similar to that of VRE. The presence of the $vanY_F$ (putative carboxypeptidase) and the $vanX_F$ (putative D,D-dipeptidase) genes suggests that at some point during growth, *P. popilliae* switches from producing the conventional D-Ala-D-Ala peptidoglycan precursor terminus to producing a peptidoglycan precursor terminating in D-Ala-D-Lac. Although the presence of the *vanZ* gene has been associated with teicoplanin resistance, *P. popilliae* ATCC 14706 is teicoplanin susceptible (1).

Marshall et al. have hypothesized that the origin of clinically relevant vancomycin resistance lies within the glycopeptide-producing organisms (11). However, the amino acid identities identified by these authors between the glycopeptide-producing organisms *Streptomyces toyocaensis* and *Amycolotopsis orientalis* and VanA VRE were 57 to 62% for VanH enzymes, 59 to 64% for DdlM and DdlN enzymes (VanA homologues), and 61 to 63% for VanX enzymes, all substantially less than the identities we found between these genes in the *vanA* gene cluster and in *P. popilliae* (Fig. 2). Furthermore, the G+C contents of the *P. popilliae* $vanH_F$, *vanF*, and $vanX_F$ genes are virtually identical to those of the homologous genes in VRE and significantly different from those of the glycopeptide resistance genes in *Streptomyces toyocaensis* and *Amycolotopsis orientalis* (Fig. 2). Therefore, the vancomycin resistance gene cluster in *P. popilliae* is more similar to that in VRE than are the gene clusters in *Streptomyces toyocaensis* and *Amycolotopsis orientalis*. Given that the G+C contents of the VRE *vanH*, *vanA*, and *vanX* genes are higher than those of the adjacent *vanR*, *vanS*, *vanY*, and *vanZ* genes, it is plausible that the *vanH*, *vanA*, and *vanX* genes have been mobilized as a unit from another source.

Recently, a new type of acquired glycopeptide resistance, termed VanE, has been described in *Enterococcus faecalis* (7). The partial sequence of VanE in *E. faecalis* BM4405 has 43% predicted amino acid identity to VanF in *P. popilliae* (7).

The *P. popilliae* isolate studied is an American Type Culture Collection type strain which was isolated from commercial spore dust and first described in the medical literature in 1961 (10). That the gene cluster present in *P. popilliae* has homology to the *vanA* (and *vanB* and *vanD*) gene cluster(s) suggests that it may have been a precursor to or have had a common ancestral origin with the *vanA* (and *vanB* and *vanD*) gene cluster(s) found in modern clinical isolates of enterococci. *P. popilliae* spores have been introduced into soil in the eastern United States as a biopesticidal powder since the early 1940s. An example of such a product, currently marketed in the United States, is Milky Spore (St. Gabriel Laboratories, Gainesville, Va.). Milky Spore is described by its producer as a product that does not affect humans or animals or contaminate well water. Once established in a lawn, Milky Spore is described as lasting 15 to 20 years. It has been suggested that spread of *P. popilliae* spores may have been accomplished by birds, insects, skunks, moles, and mice (20). Such widespread distribution of this organism may have provided the opportunity for its contact with enterococci. Furthermore, both enterococci and *P. popilliae* are able to survive for long periods in the environment; for example, we recently recovered viable *P. popilliae* from dried Japanese beetle hemolymph preserved on a microscope slide in 1945 (18). In the presence of the increasing use of oral and parenteral vancomycin in humans since the late 1970s for the treatment of *Clostridium difficile* and methicillin-resistant staphylococcal infections, respectively, and in the presence of glycopeptide usage in agriculture, the transfer of vancomycin resistance to enterococci has potentially been facilitated. Small amounts of *P. popilliae* produced in North America have been distributed in New Zealand and South America. Although we cannot prove that transfer of vancomycin resistance to enterococci occurred directly from *P. popilliae*, the evidence suggests that the use of biopesticidal preparations in agricultural practice may have an impact on bacterial resistance in human pathogens.

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