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NOTES

Isolation of Plasmid-Harboring *Serratia plymuthica* from Facultative Gut Microflora of the Tobacco Hornworm, *Manduca sexta*

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Aseptic isolation of the facultative gut microflora of the tobacco hornworm, *Manduca sexta*, yielded four microorganisms. Two were gram-positive *Bacillus* spp., one was *Serratia plymuthica*, and another was the yeast *Candida guilliermondii*. The three bacterial species were screened for extrachromosomal DNA, and *S. plymuthica* was found to have a 6.4-kilobase plasmid, which was designated pCP-1.

There is a paucity of information on the normal gut microflora of insect larvae (14), and even less is known about the presence of extrachromosomal DNA in insect gut microflora. Considering the vast number of insect species extant (estimated to be about one million [14]), the microflora of the insect gut could serve as a vast plasmid reservoir.

Manduca sexta was reared in the laboratory on nightshade (*Solanum dulcamara*) at 25°C. Before being dissected, 10-day-old larvae were washed in 95% ethanol and rinsed three times with sterile distilled water. The gut was exposed with sterile instruments, and the microflora were aseptically removed from five specimens and pooled into two 10.0-ml cultures of bovine heart infusion broth. Each culture was grown aerobically overnight, one at 25°C and the other at 37°C. Each culture was then streaked on Luria broth plates, incubated at its respective temperature, and examined after 24 and 48 h for growth.

A characteristic colony from each plate was examined by compound microscopy to determine morphology and analyzed by Gram staining. Each colony type was then stained to visualize spores and capsules and examined for motility and sugar fermentation (6).

Resistance to antibiotics and metal ions was tested by the method of Bauer et al. (2). The bacteria were classified as resistant to an antibiotic or metal ion if they grew in the presence of a disk containing 50 μg of the antibiotic (except for ampicillin, of which 100 μg was used) or 25 μg of the metal ion compound. A plasmidless *Escherichia coli* prototroph was used as a control to ensure that each antibiotic and metal ion was biologically active.

Whether the microflora removed from the *M. sexta* gut were grown at 25 or 37°C, only four distinct species were observed. Two of these were gram-positive *Bacillus* spp., one was identified as *Serratia plymuthica* on the basis of the API 20E system (Table 1), and another was the yeast *Candida guilliermondii*. All three bacteria were motile, and, although both gram-positive bacilli were sporeformers, the locations of the spores differed; the spore of one was terminal, and the spore of the other was central. All of the bacterial isolates fermented glucose, fructose, galactose, sucrose, mannose, lactose, arabinose, maltose, and xylose, but *S. plymuthica* did not ferment starch.

All three bacteria were analyzed to determine their resistances to metals and antibiotics (Tables 2 and 3). All were found to be resistant to arsenite, mercury, and silver. *S. plymuthica* was sensitive to cadmium, and of the antibiotics tested, it was resistant only to novobiocin.

When the three bacterial isolates were screened for plasmid DNA (3), only *S. plymuthica* was shown to contain a plasmid, which was found to be 6.4 kilobases upon calibration to supercoiled DNA standards (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) (Fig. 1) and was designated pCP-1. Restriction enzyme digests were run for 2.0 h, as directed by the manufacturer (IBI, New Haven, Conn., or New England BioLabs, Inc., Beverly, Mass.), and fragments were separated on a 1% agarose gel at 150 V for 2.5 h. Single digests of preparatively isolated pCP-1 (5) with *Bam*HI, *Hin*dIII, and *Eco*RI (Fig. 2) and *Pst*I, *Sph*I, and *Sma*I (data not shown) produced a single band which migrated more slowly on the agarose gel than did the uncut supercoiled plasmid, showing that the plasmid was linearized. The linearized plasmid had an apparent size of 6.4 kilobases when calibrated to lambda *Hin*dIII linear-fragment standards (Bethesda Research Laboratories).

Serratia spp. have been isolated from the gut microflora of both healthy and diseased insects, such as the honeybee, *Apis mellifera* (8, 10); the gypsy moth, *Lymantria dispar* (17); a variety of grasshoppers (4); and many other insects (11, 19). *S. plymuthica* has been isolated from insect gut microflora less frequently than *Serratia marcescens*, which is the most commonly studied member of this genus because of its pathogenicity, but Steinhaus (18) isolated it from healthy crickets, *Neombius fasciatus*, and Phaff and Miller (16) found this *Serratia* species to occur regularly in the fig wasp, *Blastophaga psenes*. It is interesting with respect to the latter insect species that *S. plymuthica* was found regularly with the yeast *C. guilliermondii*, an association noted in the present study.

It should be emphasized that the microflora isolated may not represent the major species in the gut of *M. sexta*. Conditions used for growth may have excluded other facultative microflora and certainly would have inhibited growth of anaerobes, which would be expected to be the major type of microorganism in the hindgut.

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TABLE 1. Results of the API-20E standardized test system for the differentiation of members of the family *Enterobacteriaceae* with the gram-negative bacterium isolated from *M. sexta*

Test	Reaction
Beta-galactosidase	−
Arginine dihydrolase	−
Lysine decarboxylase	−
Ornithine decarboxylase	−
Citrate	+
Hydrogen sulfide	−
Urea	−
Tryptophan deaminase	−
Indole	−
Voges-Proskauer	+
Gelatin	+
Glucose	+
Mannose	+
Rhamnose	−
Sucrose	+
Melibiose	+
Arabinose	+
Inositol	+
Sorbitol	+
Nitrate	+
Oxidase	−
Catalase	+

Novobiocin was the only antibiotic tested to which *S. plymuthica* displayed resistance. Resistance to this antibiotic has been reported to be plasmid borne in a gram-positive organism (7) and chromosomally located in a gram-negative microorganism (1). Although resistance to metals such as cadmium, mercury, and silver is commonly plasmid associated (9), recent reports have shown that determinants of resistance to mercury and cadmium are chromosomally located in some gram-positive microorganisms (15, 20). Since the plasmid from *S. plymuthica* is nonconjugative and was not curable with acridine orange (125 μg/ml), ethidium bromide (125 μg/ml), high temperature (up to 45°C), or mitomycin C (10 μg/ml) (unpublished results), it was not possible to determine what resistance genes were plasmid borne. However, the plasmid was stable in the absence of selective pressure (unpublished results).

TABLE 2. Metal resistance patterns of bacteria isolated from *M. sexta*

Metal	Resistance of[a]:		
	S. plymuthica	*Bacillus* sp. 1	*Bacillus* sp. 2
Sodium arsenite	R	R	R
Cadmium chloride	S	S	R
Sodium dichromate	R	S	S
Mercuric chloride	R	R	R
Silver nitrate	R	R	R

[a] The data presented are for a concentration of 25 μg per disk. The metals were tested on a plasmidless *E. coli* strain to ensure their biological activity. R, Resistant; S, susceptible.

TABLE 3. Antibiotic resistance patterns of bacteria isolated from *M. sexta*

Antibiotic	Resistance of[a]:		
	S. plymuthica	*Bacillus* sp. 1	*Bacillus* sp. 2
Ampicillin	S	R	S
Chloramphenicol	S	S	S
Kanamycin	S	R	R
Novobiocin	R	S	S
Spectinomycin	S	R	R
Streptomycin	S	R	R
Tetracycline	S	S	S

[a] The data presented are for an antibiotic concentration of 50 μg per disk, except for ampicillin (100 μg per disk). The antibiotics were tested on a plasmidless *E. coli* strain to ensure their biological activity. R, Resistant; S, susceptible.

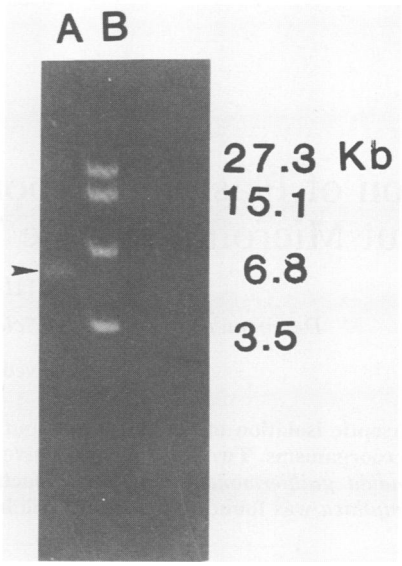

FIG. 1. Agarose gel electrophoresis of plasmid pCP-1 from *S. plymuthica*, prepared by the procedure of Birnboim and Doly (3). Lanes: A, supercoiled plasmid pCP-1 (6.4 kilobases), indicated by an arrowhead; B, supercoiled plasmid DNA standards (3.5 to 27.3 kilobases). The plasmid and standards were electrophoresed on a 0.8% agarose gel.

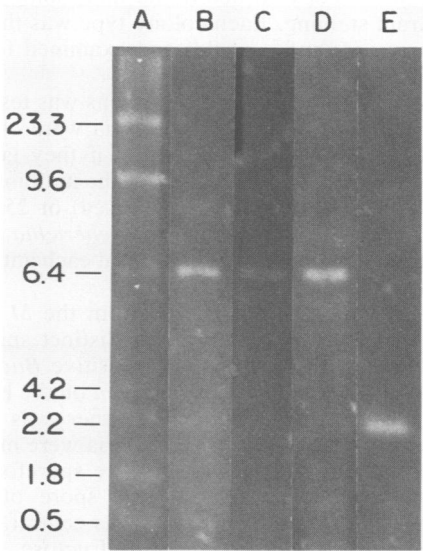

FIG. 2. Electrophoresis of restriction endonuclease fragments of preparatively isolated plasmid pCP-1. Lanes: A, lambda *Hin*dIII linear size markers; B, *Eco*RI restriction cut; C, *Bam*HI restriction cut; D, *Hin*dIII restriction cut; E, uncut plasmid pCP-1. Fragments were electrophoresed on a 1% agarose gel.

The study of extrachromosomal DNA from *Serratia* species other than *S. marcescens* has been limited (12, 13), and little if any effort has been directed toward determining whether there is a plasmid population in the gut microflora of insects. Although it is unclear what function plasmid pCP-1 serves, to our knowledge it is the first plasmid reported from *S. plymuthica* as well as the first plasmid isolated from insect gut microflora.

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