

Conjugative Plasmid Conferring Resistance to Olaquinox

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A conjugative plasmid, pOLA52, conferring resistance to the antibiotic growth promoter olaquinox has been isolated from *Escherichia coli* from swine manure. It also confers resistance to ampicillin and chloramphenicol and has a high frequency of transfer between strains of *E. coli*. Plasmid-borne olaquinox resistance has not been demonstrated before.

The synthetic chemotherapeutic agent olaquinox has found wide use as a growth promoter in pig farming. It is active against coliform bacteria (3), where it inhibits DNA synthesis (10). Resistance has been defined with a breakpoint of 64 $\mu\text{g/ml}$ (3) or 50 $\mu\text{g/ml}$ (7). Until 2000, the compound has been allowed in concentrations of up to 100 mg/kg in feed for pigs younger than 4 months.

Since the introduction of the compound in the 1980s, there has been some concern about whether resistance would arise and, if so, whether this resistance would be transferable and whether it would be linked to other resistance determinants (2, 5, 7). A survey study in Denmark has demonstrated the presence of a small fraction of olaquinox-resistant coliform bacteria in farm animals (3). Other studies have demonstrated increased resistance to olaquinox on pig farms using olaquinox. There was a slight correlation between resistance to olaquinox and resistance to chloramphenicol or ampicillin (5, 7). The aim of the present study was to find out if plasmid-bound olaquinox resistance exists.

Isolation of a resistant bacterium. Swine manure from a farm using olaquinox as a feed additive was tested for the presence of bacteria able to grow on Gould S1 medium (4) with 100 μg of olaquinox per ml. Diluted manure corresponding to 10 μl of undiluted manure gave rise to three uniform colonies that did not show the fluorescence characteristic of fluorescent pseudomonads. One of the colonies was restreaked several times on Levine EMB plates (*GIBCO Products for Microbiology: Technical Manual and Catalog*; GIBCO Laboratories, Madison, Wis.) and Luria-Bertani (LB) agar plates (9), both containing 100 μg of olaquinox per ml. Olaquinox (98% pure; ICN, Costa Mesa, Calif.) was added as a 10-mg/ml stock solution in 2.5 M NaOH; HCl was added to the final medium to counteract the high pH. All incubations were done at 37°C. The isolate was identified as *Escherichia coli* by use of the API 20E system (BioMérieux, Marcy l'Etoile, France).

Olaquinox resistance was tested with an agar dilution test, while resistance to an array of other antibiotics was tested with

Sensititre plates (1). The isolate was found to be resistant to ampicillin (MIC, >32 $\mu\text{g/ml}$), kanamycin (MIC, >64 $\mu\text{g/ml}$), chloramphenicol (MIC, >64 $\mu\text{g/ml}$), nitrofurantoin (MIC, 128 $\mu\text{g/ml}$), streptomycin (MIC, 128 $\mu\text{g/ml}$), olaquinox (MIC, 128 $\mu\text{g/ml}$), sulfamethoxazole (MIC, >512 $\mu\text{g/ml}$), trimethoprim (MIC, >32 $\mu\text{g/ml}$), and carbadox (MIC, >128 $\mu\text{g/ml}$). The strain was sensitive to apramycin (MIC, 4 $\mu\text{g/ml}$), ciprofloxacin (MIC, 0.125 $\mu\text{g/ml}$), colistin (MIC, 1 $\mu\text{g/ml}$), gentamicin (MIC, 0.5 $\mu\text{g/ml}$), nalidixic acid (MIC, 16 $\mu\text{g/ml}$), and tetracycline (MIC, 1 $\mu\text{g/ml}$). In addition, the isolate did not grow on LB agar plates containing 100 μg of rifampin per ml.

Conjugal transfer of the plasmid. Tentative resistance plasmids were transferred to *E. coli* CSH26 (8) Rif^r (resistant to rifampin at 100 $\mu\text{g/ml}$) by filter conjugation. Overnight cultures of the donor and recipient were harvested by centrifugation (6,000 $\times g$, 5 min, 4°C), washed, and resuspended in phosphate-buffered saline. Equal amounts of the two cultures were mixed and spread on sterile membrane filters (0.2- μm pore size) placed on LB agar plates. The filters were incubated at 37°C for 4 h, after which they were washed in phosphate-buffered saline. The experiment gave rise to approximately 10^{-4} transconjugants per recipient (T/R). The transconjugants had the rifampin resistance of the recipient combined with resistance to ampicillin (MIC, >32 $\mu\text{g/ml}$), chloramphenicol (MIC, 64 $\mu\text{g/ml}$), and olaquinox (MIC, 128 $\mu\text{g/ml}$). It had achieved intermediate resistance to nitrofurantoin (MIC, 64 $\mu\text{g/ml}$) relative to the recipient and was sensitive to kanamycin, streptomycin, sulfamethoxazole, trimethoprim, and carbadox. The recipient strain did not grow on plates containing 100 μg of olaquinox per ml. Some of the resistance determinants of the isolate, including olaquinox resistance, had thus been transferred to *E. coli* CSH26, while others had not. This is consistent with earlier observations (5) that olaquinox resistance can be linked to chloramphenicol resistance. In this case, it is also linked to ampicillin resistance and—to some extent—nitrofurantoin resistance.

Plasmid DNA preparations (6) showed that the transconjugants had received one of the plasmid bands of the donor (data not shown). This plasmid was named pOLA52.

Restriction map. A restriction map of the plasmid has been constructed (Fig. 1) by use of the enzymes *EcoRI*, *BglII*, *HindIII*, *BamHI*, and *SmaI*. A QIAprep Spin Miniprep Kit and a QIAGEN Plasmid Midi Kit were used for purification of plasmid DNA (QIAGEN GmbH, Hilden, Germany). In order

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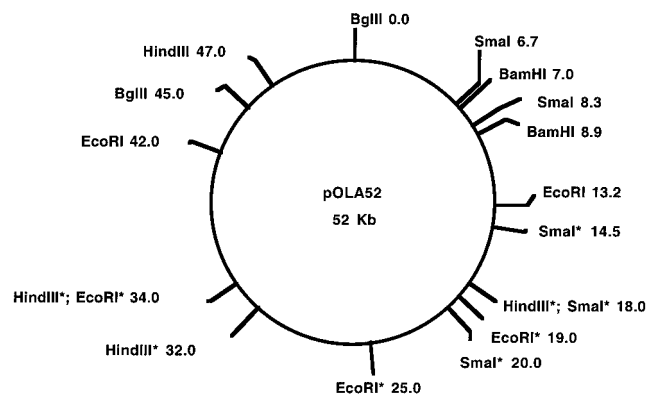


FIG. 1. Restriction map of pOLA52. Sites marked with asterisks have not been located exactly. The numbers following the names of the restriction enzymes are distances (in kilobases) from the *Bgl*II site defined to be the numbering starting point.

to improve the separation of large DNA fragments, these were subjected to pulsed-field gel electrophoresis with the Gene Navigator System (with a version 18-1019-19 electrophoresis unit and a HEX electrode) from Pharmacia LKB Biotechnology AB, Uppsala, Sweden.

Frequency of conjugal transfer. Conjugal transfer of pOLA52 between *E. coli* CSH26 Rif^r and *E. coli* CSH26 Nal^r (resistant to nalidixic acid at 100 µg/ml) was tested further. The frequency of transfer was 0.0069 to 0.055 T/R (mean, 0.026 T/R; standard deviation, 0.015; $n = 9$). Fifteen transconjugants were tested for resistance to olaquinox (100 µg/ml), rifampin (100 µg/ml), nalidixic acid (100 µg/ml), ampicillin (100 µg/ml), and chloramphenicol (20 µg/ml). Fourteen of the transconjugants tested had the antibiotic resistance profile expected (nalidixic acid, ampicillin, chloramphenicol, and olaquinox), but in one case, the ampicillin resistance was lost. Plasmid DNA was purified from eight transconjugants by use of QIAprep spin columns (QIAGEN GmbH). The DNA was eluted from the spin columns with water preheated to 70°C. Aliquots of the plasmid DNA preparations were digested with *Eco*RI, followed by gel electrophoresis. The gels were photographed in a Bio-Rad Gel Doc 1000 setup controlled with the Quantity One software, version 4.0.3. Plasmid DNAs from the transconjugants gave identical band patterns on agarose gels, except for the strain that had lost its ampicillin resistance. A similar loss of ampicillin resistance and an altered band pattern were found in transconjugants from several preliminary conjugation experiments. Figure 2 shows examples of band patterns from the donor and from transconjugants with and without loss of ampicillin resistance.

Concluding remarks. In this work, we have isolated a highly conjugative plasmid conferring resistance to olaquinox, ampicillin, and—to some extent—chloramphenicol and nitrofurantoin. Plasmid-borne olaquinox resistance has not been demonstrated before. The fact that the plasmid was readily found in pig manure shows that there is a potential for spread

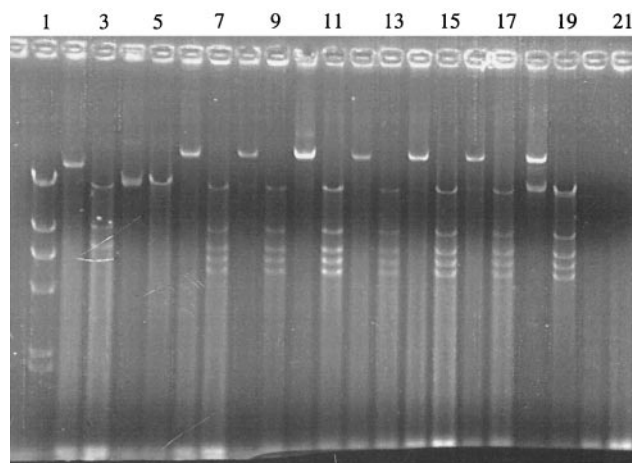


FIG. 2. Plasmid preparations from strains with and without pOLA52. All preparations were run with and without prior digestion with *Eco*RI. Lanes: 1, λ DNA digested with *Hind*III; 2 to 5, plasmid preparations from two transconjugants that had lost the ampicillin resistance of the plasmid (each is shown undigested and digested); 6 to 17, plasmid preparations from six transconjugants (each is shown undigested and digested); 18 and 19, plasmid preparation from the donor, undigested and digested; 20 and 21, "plasmid preparation" from the recipient (no plasmids), undigested and digested.

of olaquinox resistance among coliform bacteria and that this can be accompanied by the spread of other antibiotic resistance determinants.

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