APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1993, p. 2531–2537 Vol. 59, No. 8
0099-2240/93/082531-07$02.00/0
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Copper-Resistant Enteric Bacteria from United Kingdom and Australian Piggeries

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Received 22 March 1993/Accepted 29 May 1993

Thirty-three enteric isolates from Australian (*Escherichia coli* only) and United Kingdom (U.K.) (*Salmonella* sp., *Citrobacter* spp., and *E. coli*) piggeries were characterized with respect to their copper resistance. The copper resistance phenotypes of four new Australian *E. coli* isolates were comparable with that of the previously studied *E. coli* K-12 strain ED8739(pRJ1004), in that the resistance level in rich media was high (up to 18 mM $CuSO_4$) and resistance was inducible. Copper resistance was transferable by conjugation from the new Australian isolates to *E. coli* K-12 recipients. DNA similarity between the new Australian isolates and the *pco* copper resistance determinant located on plasmid pRJ1004 was strong as measured by DNA-DNA hybridization; however, the copper resistance plasmids were nonidentical as indicated by the presence of restriction fragment length polymorphisms between the plasmids. DNA-DNA hybridization and polymerase chain reaction analysis demonstrated DNA homology between the *pco* determinant and DNA from the U.K. *E. coli*, *Salmonella* sp., and *Citrobacter freundii* isolates. However, the copper resistance level and inducibility were variable among the U.K. strains. Of the U.K. *E. coli* isolates, 1 demonstrated a high level of copper resistance, 4 exhibited intermediate resistance, and 16 showed a low level of copper resistance; all of these resistances were expressed constitutively. A single U.K. *C. freundii* isolate had a high level of copper resistance, inducible by subtoxic levels of copper. Transconjugants from one *E. coli* and one *C. freundii* donor, with *E. coli* K-12 strain UB1637 as a recipient, showed copper resistance levels and inducibility of resistance which differed from that expressed from plasmid pRJ1004. We conclude that closely related resistance determinants in nonidentical plasmids are responsible for copper resistance in enteric bacteria isolated at separate geographic locations.

Copper has been used as the active ingredient in bacteriocides and fungicides on fruits and vegetables for more than 100 years. Since the mid-1980s, copper-resistant bacterial pathogens have been detected repeatedly (7, 10). However, copper resistance was the most frequently found resistance in bacteria from pre-antibiotic era isolates (17). The copper resistance determinant from a Florida *Xanthomonas campestris* pv. vesicatoria isolate (a pathogen of pepper and tomato plants) was found on a conjugative plasmid pXVCu1 (25). Bender and Cooksey (1a) demonstrated that another pathogen of tomatoes, *Pseudomonas syringae* pv. tomato, carries a plasmid containing a copper resistance determinant, named *cop* (2).

Copper and antibiotics have been used as growth promoters in pig diets for at least 40 years (5, 15). This practice has continued because pigs fed such supplemented diets may demonstrate an improved average daily weight gain and food conversion efficiency (5, 9, 15). It has been proposed that copper elicits an antibacterial effect on the bacteria of the pig gut (22, 25). We are interested in the nature of copper resistance determinants in bacteria, whether they are related, and how widespread they are.

Plasmid pRJ1004 was isolated from an *Escherichia coli* strain from the feces of pigs fed copper sulfate at approximately 180 ppm (26). The plasmid confers on the host *E. coli* K-12 strain ED8739 a 2.5- to 3-fold increase in tolerance to copper on Luria broth agar supplemented with $CuSO_4$, an increase in resistance to silver (8), and an increased sensitivity to zinc (26). Plasmid pRJ1004 carries no other known antibiotic or heavy metal resistance markers. A 12.5-kb *Hin*dIII fragment from pRJ1004 was shown to contain the plasmid-borne copper resistance determinant (*pco*) and confer copper resistance in the host *E. coli* HB101 almost equivalent to that of ED8739(pRJ1004) (18).

We have isolated other enteric organisms from similar but geographically distinct environments and characterized their copper resistance phenotypes. Colony formation on $CuSO_4$-supplemented agar and induction assays, with subinhibitory concentrations of $CuSO_4$, identified the enteric isolates in this study as having either normal (wild-type) copper sensitivity or copper resistance. The resistant isolates were investigated to determine their similarity to the *pco* determinant and to ascertain how well the resistance determinant has been conserved geographically and temporally. The results presented here show significant DNA homologies between the *pco* determinant and DNA from other copper-resistant enteric bacteria from Australia and the United Kingdom (U.K.).

MATERIALS AND METHODS

Bacterial strains. The sources and characteristics of the bacterial strains used are listed in Tables 1 and 2. The newly isolated strains were obtained from fecal swabs taken from pigs housed in Australia and in the U.K.

Copper induction assay. To test for induction of growth

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TABLE 1. Strains used

Strain or species	Phenotype or genotype	Source or reference
ED8739	*metB gal lac supE supF hsdR hsdM*	4
GME008	ED8739, spontaneous nalidixic acid-resistant mutant	This study
UB1637	*his lys trp recA56 strA* (*rpsL*)	14
ED8739(pRJ1004)	pRJ1004, copper resistant	27
JW001	Copper-resistant transconjugant from GME008 × 1988-1	This study
JW002	Copper-resistant transconjugant from GME008 × 1988-8	This study
UB1637(pRJ1004)	Copper-resistant transconjugant from UB1637 × ED8739(pRJ1004)	This study
JW101	Copper-resistant transconjugant from UB1637 × JW001	This study
JW102	Copper-resistant transconjugant from UB1637 × JW002	This study
C. freundii	Wild-type, copper tolerant	NCTC 9750

resistance by subinhibitory levels of $CuSO_4$, the strains were grown overnight in Luria broth. The following day, 40 μl of each culture was inoculated into 2 ml of fresh Luria broth containing 0, 0.5, or 2.0 mM added $CuSO_4$ and was incubated at 37°C for 3 h with shaking. The amount of culture remaining on a 1-cm length of inoculating wire after being dipped into 2 ml of broth was streaked on a gradient agar plate; the gradient ranged from 0 to 20 mM $CuSO_4$.

The $CuSO_4$ gradient plates were prepared with a modification of the method of Szybalski and Bryson (cited in reference 16). Twenty-five milliliters of Luria broth agar supplemented with 20 mM $CuSO_4$ (pH adjusted to 7.5) was poured into 10-cm square petri dishes and allowed to set with the dishes inclined at an angle of approximately 4°. The agar was dried for 20 min in a laminar flow cabinet, and then a second layer of 25 ml of Luria broth agar without added $CuSO_4$ was layered on top while the plate lay horizontally. These gradient plates were poured and used on the same day. Each growth measurement was performed in triplicate.

Bacterial conjugation. (i) Australian isolates. The newly isolated donor strains and the recipient *E. coli* GME008 were inoculated into Luria broth and incubated overnight at 37°C with shaking. Both strains were then inoculated into fresh Luria broth (125 μl into 5 ml) and were incubated for 3 h at 37°C with shaking. The donor and recipient were then combined in a ratio of 1:3, and conjugation was allowed to proceed at 37°C for a period of 3 to 24 h in static culture. Aliquots were plated out at regular time intervals on the selection plates of Luria broth agar supplemented with 10 mM $CuSO_4$ and nalidixic acid (30 μg/ml). As controls, the individual strains were plated out on the same selection plates. Putative transconjugants were purified by streaking on fresh selection plates.

TABLE 2. Copper-resistant enteric bacterial isolates

Strain	Species identification	Source
1979 isolates: TJ48, TJ51, TJ55, and TJ57	*E. coli*	Victoria, Australia
1988 isolates		
1988-1, -2, -3, -4, -5, -6, and -7	*E. coli*	Shropshire, U.K.
1988-8, -9, and -10	*C. freundii*	Shropshire, U.K.
1989 isolates		
1989-E, -J, -K, -L, and -R	*E. coli*	Shropshire, U.K.
1989-C, -H, and -O	*C. freundii*	Shropshire, U.K.
1989-S	*Salmonella* sp.	Shropshire, U.K.
1990 isolates		
1990-1, -2, -5, -9, and -10	*E. coli*	Shropshire, U.K.
1990-11, -14, and -15	*E. coli*	Humberside, U.K.
1990-17	*E. coli*	Lothian, U.K.
1990-4	*C. freundii*	Shropshire, U.K.

(ii) U.K. isolates. Initial experiments with the U.K. isolates showed that they did not transfer readily in broth matings. In order to improve the frequency of conjugal transfer, filter matings were used. The donor and the recipient, *E. coli* GME008 or UB1637, were inoculated into Luria broth and were incubated overnight at 37°C with shaking. Both strains were then inoculated into fresh Luria broth (125 μl into 5 ml) and were incubated at 37°C with shaking for 3 h. Donor (1-ml) and recipient (2-ml) cultures were combined in a sterile 5-ml disposable syringe and were mixed and transferred onto a sterile cellulose acetate filter (2.5-cm diameter, pore size of 0.45 μm) held in a sterile filter holder. The filter was aseptically removed and placed onto a dried Luria broth agar plate with the bacteria uppermost. The filters on the agar plates were incubated at 37°C for periods of between 24 and 48 h. The bacteria were then resuspended by swirling the filter in 2 ml of Luria broth, and aliquots were plated out onto 10 mM $CuSO_4$ agar containing 30 μg of nalidixic acid per ml (for GME008) or 100 μg of streptomycin per ml (for UB1637). As controls, the individual strains were plated out onto the same selection plates. Putative transconjugants were purified by streaking on fresh selection plates.

Preparation of plasmid DNA. The method used for extracting plasmid DNA from the Australian isolates was a modification of the alkaline lysis method of Birnboim and Doly (3). After the first ethanol precipitation of plasmid DNA, the DNA was precipitated a second time by the addition of one-third volume of 30% polyethylene glycol 6000 in 1.8 M NaCl.

Preparation of bacterial genomic DNA. Bacteria were grown overnight in 5 ml of Luria broth. The cells were pelleted at 5,500 × *g* at 4°C for 10 min. After the cell pellet was resuspended in 0.25 ml of 10 mM Tris-Cl–1 mM EDTA buffer (pH 8.0), 0.05 ml of lysozyme (freshly dissolved at 10 mg/ml in the same buffer) was added and the mixture was incubated at 37°C for 20 min. This was followed by the addition of 0.02 ml of 25% sodium dodecyl sulfate (SDS) with incubation at 60°C for 15 min. The mixture was quickly cooled on ice before 0.08 ml of 5 M sodium perchlorate was added. The DNA solution was treated with a buffered phenol-chloroform-isoamyl alcohol (24:24:1) extraction followed by two extractions with chloroform-isoamyl alcohol

(24:1). The DNA was precipitated at −20°C by the addition of 1/10 volume of 3 M sodium acetate and 2 volumes of ethanol. The pellet was rinsed with 70% alcohol, dried under vacuum, and resuspended in sterile water.

DNA-DNA hybridization. A modification of the method of Southern (23) was used for DNA-DNA hybridization analysis. DNA fragments separated by horizontal gel electrophoresis (in 0.8% agarose in 90 mM Tris-borate [pH 8.3]–2.5 mM EDTA–0.05 μg of ethidium bromide per ml) were denatured and transferred to a nitrocellulose membrane (Schleicher and Schuell) (21). The 6.2-kb *Pvu*I fragment from the plasmid pPA87 (in which the 12.5-kb *Hin*dIII fragment containing *pco* is cloned in a tandem repeat of pBR322 [18]) was purified by electrophoresis in low-gelling-temperature agarose and adsorption onto a DEAE-cellulose membrane (21) and then was labelled with [α-^{32}P]dATP with an OLK oligonucleotide labelling kit from Bresa Pty. Ltd. (Adelaide, Australia) according to the manufacturer's instructions. Unincorporated nucleotides were removed on a Sephadex spun column, and the nitrocellulose membrane was prehybridized, hybridized with the labelled probe, and washed (21). High-stringency washes were done three times, the final wash being with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 65°C. Probes were removed from filters by heating the filters to 95°C in 0.1× SSC for approximately 30 min, and the filters were air dried. The filters were checked for removal of the probe by reexposure to X-ray film. Once clean, the filters could be prehybridized and reprobed.

PCR. Oligonucleotide primers were chosen from the *pco* sequence data (1), and were prepared by Alta Bioscience (Birmingham, United Kingdom). The sequences of the polymerase chain reaction (PCR) primers used are 5′-GATATC TGACTCCCTGGCAAAA-3′ (primer X), 5′-TTAAGCCCT TTGATTTTACGG-3′ (primer 2), 5′-CTCTGAGACCATG CAGGAGT-3′ (primer 11), 5′-TTTCCCGAATTAGTCC ATCA-3′ (primer 18), 5′-CTTCAAATGGACAGTCTT-3′ (primer 10), 5′-CCCGAGCTTGTTGAGTGCA-3′ (primer 8), and 5′-CAAAGCAATCTTCGACGACCG-3′ (primer 24).

A 50-μl reaction mixture contained 100 pmol of each primer, 200 mM each deoxynucleoside triphosphate, approximately 1 μg of template DNA, 4.2 U of thermostable DNA polymerase (Tub; Amersham International plc, Amersham, United Kingdom), and the reaction buffer provided by Amersham; controls without template were also run. The reaction mixture was sealed with 100 μl of mineral oil. The PCR was typically run for 30 cycles in an automated thermocycler (Techne PHC-2) with the following cycle profile: 93°C for 1.75 min, 55°C for 1.5 min, and 72°C for 2.0 min. PCR samples (5 μl per lane) were run on 0.8% agarose gels in 90 mM Tris-borate (pH 8.3)–2.5 mM EDTA–0.05 μg of ethidium bromide per ml and were visualized on a UV transilluminator.

RESULTS

Australian isolates. The four isolates from a single pig farm in Australia (TJ48, TJ51, TJ55, and TJ57) were able to form colonies on Luria broth agar plus 18 mM $CuSO_4$. The level of resistance on copper gradient plates was increased (induced) by pregrowth in subinhibitory concentrations of $CuSO_4$, although strain TJ51 was either noninducible or possibly marginally inducible (Table 3). Plasmids from the four TJ strains were shown to be conjugative, and the copper resistance determinant in each of the TJ strains was apparently linked to a tetracycline resistance marker, since after primary selection for either the copper or tetracycline marker in transconjugants, 100% cotransfer of the second marker was found.

TABLE 3. Growth of control and representative copper-resistant bacterial isolates on copper gradient plates

Strain	Growth (cm) on 0–20 mM $CuSO_4$ gradient plates with[a]:		
	0 mM $CuSO_4$	0.5 mM $CuSO_4$	2 mM $CuSO_4$
E. coli			
Control ED8739	4.2 ± 0.1	4.4 ± 0.2	4.4 ± 0.2
ED8739(pRJ1004)	7.9 ± 0.5	9.6 ± 0.3	10.0 ± 0.0
Australian isolates			
TJ48	7.2 ± 0.1	8.1 ± 0.2	8.9 ± 0.6
TJ51	8.3 ± 0.8	8.4 ± 0.7	8.5 ± 0.7
TJ55	8.1 ± 0.3	8.9 ± 0.5	9.4 ± 0.5
TJ57	6.6 ± 0.3	7.1 ± 0.4	7.8 ± 0.4
U.K. isolates			
Group 1 1988-1	9.5 ± 0.4	9.5 ± 0.4	9.5 ± 0.2
Group 2[b] 1988-6	8.1 ± 0.7	8.3 ± 0.6	8.5 ± 0.6
Group 3[b] 1989-L	5.5 ± 0.2	5.5 ± 0.2	5.5 ± 0.2
C. freundii			
Control 345	7.1 ± 0.1	7.1 ± 0.1	7.1 ± 0.1
Group 1 1988-8	8.2 ± 0.5	9.1 ± 0.4	9.7 ± 0.3
Group 2[b] 1989-O	6.7 ± 0.1	6.7 ± 0.1	6.7 ± 0.1
Salmonella sp., 1989-S	7.7 ± 0.1	7.7 ± 0.1	7.7 ± 0.1

[a] Growth was measured as the mean length of bacterial growth (± standard error of the mean; $n = 3$) in centimeters along the Cu^{2+} gradient from the zero concentration end. Zero, 0.5, or 2 mM $CuSO_4$ was added to the pregrowth medium as indicated.

[b] Other U.K. copper-resistant bacterial isolates which belong to *E. coli* group 2 include 1988-5, 1989-E, and 1989-R; others which belong to *E. coli* group 3 include 1988-2, 1988-3, 1988-4, 1988-7, 1989-J, 1989-K, 1990-1, 1990-2, 1990-5, 1990-9, 1990-10, 1990-11, 1990-14, 1990-15, and 1990-17; and others which belong to *C. freundii* group 2 include 1988-9, 1988-10, 1989-C, 1989-H, and 1990-4.

The 6.2-kb *Pvu*I fragment from the *pco* determinant of pRJ1004 demonstrated strong homology to total plasmid preparations from each of the TJ strains, which were cut with *Eco*RI, *Eco*RV, or *Hin*dIII in Southern hybridization experiments under high-stringency washing conditions (see Fig. 2, left panel). Hybridizing DNA fragments completely internal to *pco* (the 3.0-kb *Eco*RI and 2.9-kb *Eco*RV fragments; Fig. 1) were present in pRJ1004 and all four plasmid DNAs tested. However, bands which overlapped with the region outside that was defined genetically as *pco* (18, 20) differed between strains. The *pco* determinant is completely contained within a 12.5-kb *Hin*dIII fragment in pRJ1004, and this is shown to hybridize to the *Pvu*I probe fragment in Fig. 1. The corresponding *Hin*dIII fragments from the TJ strains were smaller than that in pRJ1004: those in TJ48 and TJ55 were of similar size (ca. 10.5 kb), that in TJ57 was approximately 9.5 kb, and that from TJ51 was the smallest fragment (ca. 7 to 8 kb). The *Pvu*I probe fragment hybridized only to *Eco*RI and *Eco*RV fragments, which are internal to *pco* in pRJ1004 or which overlap the right end of the determinant and contain both *pco* sequences and flanking sequences outside the region defined genetically as *pco*. As shown in Fig. 2, these *Eco*RI and *Eco*RV junction fragments are a different size in pRJ1004 (5.1 kb for *Eco*RI and 3.4 kb for *Eco*RV) from those in the TJ strains, all of which are similar

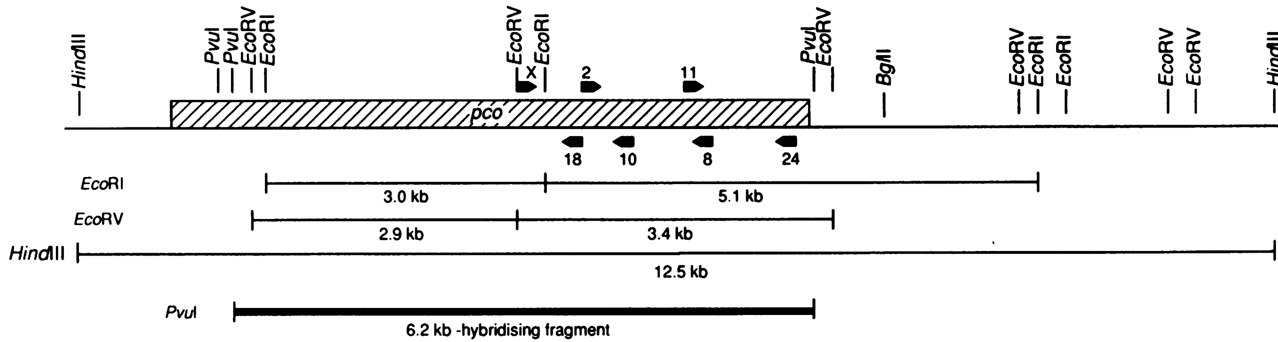

FIG. 1. Map of the *Hin*dIII fragment of pRJ1004 containing the *pco* region, showing the relevant restriction sites and locations of the oligodeoxyribonucleotide primers used for PCR analysis of the U.K. isolates. Sizes of the PCR products generated by the primer pairs: X and 18, 726 bp; 2 and 10, 526 bp; 11 and 8, 333 bp; X and 24, 3,008 bp.

or identical in size (4.0 kb for *Eco*RI and 5.5 kb for *Eco*RV). The difference in sizes of these junction fragments suggests variability in the positions of the *Eco*RI and *Eco*RV restriction sites flanking but outside of the *pco* determinant. The *Bgl*II-*Hin*dIII fragment (from the right of *pco* in plasmid pPA87; approximately 4 kb), which does not contain *pco* (Fig. 1), was hybridized to the same blot (Fig. 2, right panel). After high-stringency washing, bands of homology to this flanking region of pRJ1004 were visible in the DNA. In addition, this *Bgl*II-*Hin*dIII fragment hybridized weakly to

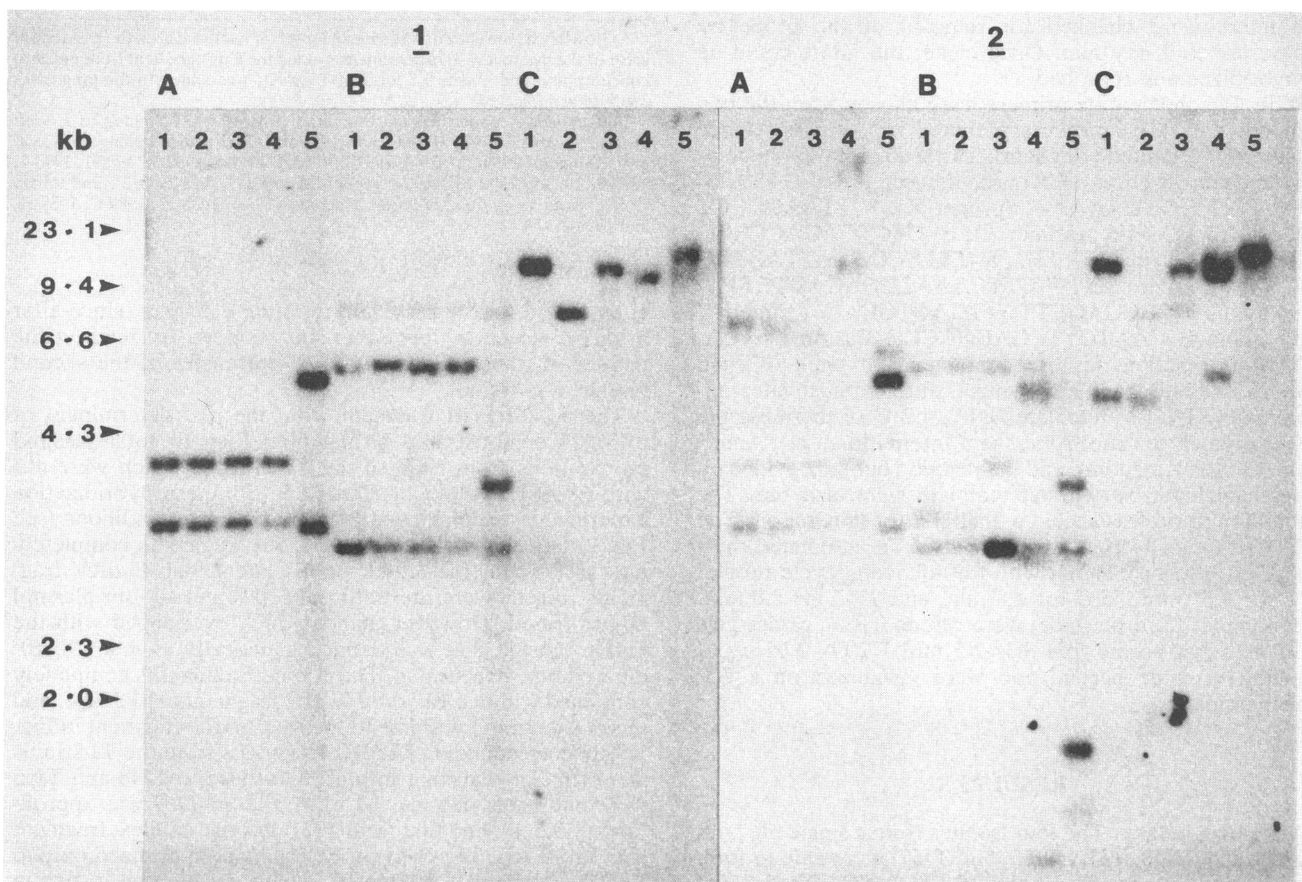

FIG. 2. Autoradiographs of Southern blots of total plasmid DNA from the Australian isolates TJ48, TJ51, TJ55, and TJ57 and plasmid pRJ1004 DNA. The DNA was digested with the restriction enzymes *Eco*RI (A), *Eco*RV (B), or *Hin*dIII (C), electrophoresed through agarose gels, blotted onto nitrocellulose filters, and probed with the ^{32}P-labelled purified 6.2-kb *Pvu*I probe (1, left panel), which contains *pco*, and, after the removal of this probe, the DNA was reprobed with a ^{32}P-labelled, purified *Bgl*II-*Hin*dIII (approximately 4-kb) fragment (2, right panel) from the right of *pco* in plasmid pPA87, which does not contain *pco*. Fragment sizes of standards are given in kilobase pairs (kb). Lanes: 1, TJ48; 2, TJ51; 3, TJ55; 4, TJ57; 5, pRJ1004.

the *pco* portion of plasmid pRJ1004 and to plasmid DNA from the TJ strains. The sizes of these bands were consistent with blotting to the *pco* determinants in each case. That this fragment also hybridized to the *pco* sequences of plasmid pRJ1004 suggests that two closely related and adjacent sequences are in the *pco* region of pRJ1004. Results from hybridizing the *pco* probe to genomic DNA of the control strain ED8739 did not show any significant bands of homology, suggesting that there is no *pco* homolog on the genome of *E. coli*.

U.K. isolates. The 21 *E. coli* isolates could be separated into three groups by resistance levels (Table 3) and by colony-forming ability (CFU per milliliter) on Luria broth agar containing increasing concentrations of $CuSO_4$ (data not shown). Isolate 1988-1 (group 1) showed the highest level of resistance, and the level of resistance was not increased by pregrowth in subinhibitory concentrations of copper. Group 2 strains exhibited an intermediate level of resistance to copper, which was only marginally increased, if at all, after pregrowth in subinhibitory concentrations of copper. Group 2 strains showed 10- to 100-fold lower colony formation rates than 1988-1 at high copper concentrations (data not shown). The low resistance level of group 3 strains was not altered by pregrowth in subinhibitory concentrations of copper, and these strains showed 10^2- to 10^5-fold fewer colonies formed at high copper concentrations in survival assays than did strain 1988-1 (data not shown). Therefore, induction of the copper resistance phenotype in the U.K. *E. coli* isolates by pregrowth of the strains in subinhibitory concentrations of copper was generally not found. The *Salmonella* sp. isolate had an intermediate copper resistance level and was not inducible.

Of the seven U.K. *Citrobacter freundii* isolates, six displayed a copper resistance phenotype similar to that of the copper-tolerant reference strain *C. freundii* 345 (Table 3). The resistance level of these strains was not increased after pregrowth in subinhibitory concentrations of copper. *C. freundii* isolate 1988-8 tolerated up to approximately 20 mM $CuSO_4$, and the level of resistance achieved on copper gradient plates was increased by pregrowth in copper; i.e., resistance was inducible.

Initial conjugation experiments with the U.K. *E. coli* and *C. freundii* isolates as donors showed a very low frequency of transfer of the copper resistance determinants (approximately 1×10^{-9} transconjugants per donor cell). No spontaneous resistant mutant isolates of GME008 were obtained from 50 separate control plates (Luria broth agar plus 8 or 10 mM added $CuSO_4$) on which 10^6 recipient GME008 cells were plated per plate. In further attempts to isolate strains with chromosomal resistance to copper, 50×10^9 cells were spread on copper-containing plates, but no spontaneous copper-resistant isolates were observed. Spontaneous copper-resistant isolates also have not been obtained after the plating and purification of GME008 on Luria broth agar containing incremental concentrations of added $CuSO_4$ (17a). Transconjugant derivatives of *E. coli* GME008 were obtained from the U.K. donor strains *E. coli* 1988-1 and *C. freundii* 1988-8 (JW001 and JW002, respectively). Each had the same level of resistance, which was similar to that of ED8739(pRJ1004) (Table 4). Colony-forming ability on Luria broth agar showed that ED8739 had a copper sensitivity similar to that of GME008. The copper resistance phenotype of isolate *E. coli* 1988-1 was not inducible when tested on gradient plates. However, the derived transconjugant JW001 showed an inducible copper resistance phenotype under the same conditions.

TABLE 4. Growth of recipient strains and transconjugants on copper gradient plates

Strain	Growth (cm) on 0–20 mM $CuSO_4$ gradient plates with[a]:		
	0 mM $CuSO_4$	0.5 mM $CuSO_4$	2 mM $CuSO_4$
GME008	4.2 ± 0.1	4.4 ± 0.2	4.4 ± 0.2
ED8739(pRJ1004)	7.9 ± 0.5	9.6 ± 0.3	10.0 ± 0.0
JW001	8.0 ± 0.2	8.9 ± 0.5	9.2 ± 0.3
JW002	8.1 ± 0.4	8.7 ± 0.5	9.3 ± 0.3
UB1637	2.9 ± 0.05	2.9 ± 0.05	2.9 ± 0.05
UB1637(pRJ1004)	4.3 ± 0.1	9.0 ± 0.5	10.0 ± 0.0
JW101	7.0 ± 0.4	8.0 ± 0.6	9.0 ± 0.4
JW102	4.3 ± 0.3	8.0 ± 0.5	8.9 ± 0.5

[a] Growth was measured as the mean length of bacterial growth (± standard error of the mean; $n = 3$) in centimeters along the Cu^{2+} gradient from the zero concentration end. Zero, 0.5, or 2 mM $CuSO_4$ was added to the pregrowth medium as indicated.

The copper resistance determinants from the U.K. isolates 1988-1 and 1988-8 were further conjugated from strain GME008 into *E. coli* UB1637 (JW101 and JW102). Copper resistance encoded by plasmid pRJ1004 in UB1637 was more strongly induced after pregrowth of the transconjugant in Luria broth with 2 mM $CuSO_4$ than that in either of the transconjugants JW101 and JW102 (Table 4). However, in the absence of induction with added $CuSO_4$, the resistance conferred on the host UB1637 by the determinant from isolate 1988-1 was greater than that for the determinants from either pRJ1004 or isolate 1988-8. Attempts to isolate plasmid DNA from these U.K. strains and the transconjugants were unsuccessful.

DNA-DNA hybridization experiments with total DNA from the U.K. isolates 1988-1, 1988-3, and 1988-8 revealed strong similarity to the *pco* determinant, and restriction nuclease fragment length measurements suggest that sequences flanking *pco* differ from those in pRJ1004 and among the U.K. strains (data not shown). PCR analysis with total DNA from the 29 U.K. isolates and *pco* primers showed apparently identical products on ethidium bromide-stained agarose gels for 16 of the 21 U.K. *E. coli* strains, for 6 of the 7 U.K. *C. freundii* strains, and for the U.K. *Salmonella* sp. isolate. No products were detected from PCR analysis of genomic DNA of the control strains ED8739 and *C. freundii* 345 when the *pco* primer pair X and 18 was used (Fig. 1). *Sau*3AI restriction enzyme digest analysis of PCR products amplified from the DNA of the U.K. strains 1988-1, 1988-3, and 1988-8 and plasmid pRJ1004 showed identical patterns of bands on acrylamide gels (Fig. 3). Preliminary DNA sequence data of a PCR fragment from strain 1988-8 demonstrated 94% identity to the *pco* sequence over 114 bp (data not shown).

DISCUSSION

We have shown strong similarity between the previously characterized *pco* copper resistance determinant originally detected on plasmid pRJ1004, which was isolated in Australia in 1979 (19, 20, 26), and four additional copper-resistant *E. coli* strains isolated in Australia in the same year. The four strains contain conjugative plasmids which confer copper resistance. The four *E. coli* strains contain sequences showing close similarity to *pco*, which are flanked (at least on one side) by sequences which differ from those in pRJ1004. The copper resistance in these strains is conferred by at least

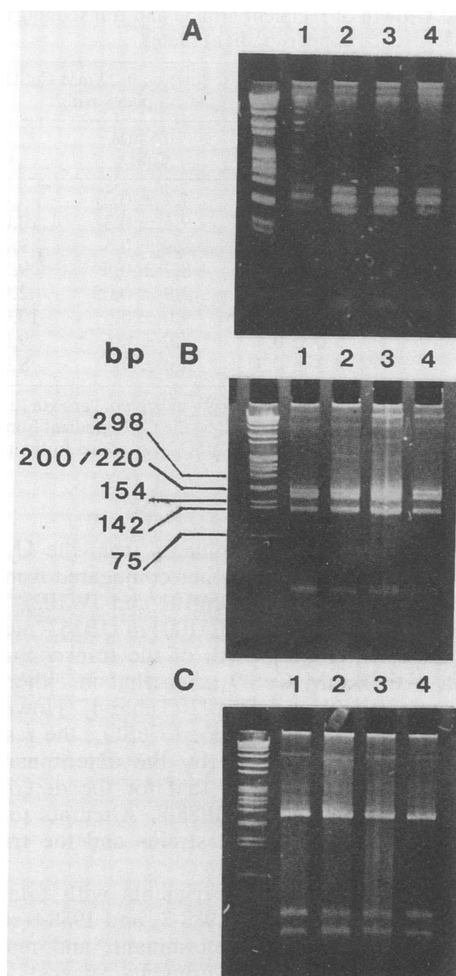

FIG. 3. DNA fragments produced after digestion of PCR products with the restriction enzyme *Sau*3AI. The PCR products were produced by amplification of template DNA with the *pco* primer pairs X and 18 (A), 2 and 10 (B), and 11 and 8 (C). Molecular size markers are in the left-hand lanes. Lanes: 1, U.K. *E. coli* 1988-1; 2, U.K. *E. coli* 1988-3; 3, U.K. *C. freundii* 1988-8; 4, pRJ1004.

three different, albeit related, conjugative plasmids, namely, pRJ1004, the plasmid present in TJ51, and the plasmids present in the remaining TJ strains tested. Homology has also been seen between *pco* and a number of *E. coli* strains isolated from U.K. pig feces in 1988. The PCR data show conservation of restriction nuclease fragment sizes. In one of these strains, there was 94% identity of nucleotide sequence across a short region. These data show that internal sequences within *pco* are conserved in many of the strains, but DNA hybridization data indicate that the sequences flanking *pco* vary between the strains.

The PCR and hybridization analysis of the U.K. and Australian strains indicate that the *pco* determinant is widespread in copper-resistant enteric bacteria isolated from pig feces but that the determinant is found in a variety of DNA molecules, some of which are nonidentical conjugative plasmids. The determinant has not been spread by the recent conjugative transfer of a single plasmid.

The variable phenotypic expression of the U.K. copper resistance determinants in different genetic backgrounds indicates that genetic information other than the resistance determinant itself contributes to the expression of resistance. This is highlighted by the U.K. *E. coli* isolate 1988-1, which shows constitutive copper resistance, whereas transconjugants after transfer of *pco* into the *E. coli* K-12 derivatives GME008 and UB1637 were inducible for copper resistance. Chromosomal genes such as the regulatory gene *cutR* have been postulated to be involved in both the mechanism of resistance and the regulation of its expression (7, 20). We were unable to detect *pco*-related genes in the *E. coli* chromosome by DNA hybridization.

Additional copper resistance determinants are known. The *cop* determinant from plasmid pPT23D of *P. syringae* pv. tomato (1a, 2) shows no hybridization to *pco* by Southern analysis, although DNA sequence data (1) show considerable similarity between the *pco* and *cop* protein product sequences. Cooksey et al. (12) demonstrated homology between the *cop* plasmid genes of *P. syringae* pv. tomato and the chromosomal DNA of copper-sensitive *Pseudomonas* isolates. They suggest that the copper resistance operon evolved from chromosomal genes, which perhaps have some other cellular function. The mechanism of *cop*-encoded copper resistance is sequestration of copper and higher accumulation (11), whereas that encoded by *pco* is energy-dependent export and lower accumulation (18, 20).

The data presented here show that a *pco*-like determinant is also present and functional in *C. freundii* and a *Salmonella* sp. and show DNA homology between the copper resistance determinants in these species and *pco*. The proposed mechanism of copper resistance in *E. coli* requires that both plasmid and chromosomal functions contribute to resistance in an integrated fashion, such that resistance to high concentrations of copper can be expressed while homeostasis of intracellular copper concentrations is maintained within physiological requirements for metalloenzyme function. The range of bacterial hosts in which the *pco* determinant could function might be limited to those genera closely related to *E. coli*, such as *Citrobacter*, *Salmonella*, and *Shigella*. Studies on the DNA extracted from copper-resistant *Proteus* spp., which were isolated in the same screening program, did not demonstrate DNA homology to the *pco* determinant by high-stringency hybridization (data not shown).

We propose that *pco* and related sequences have transferred between strains now found in geographically distinct locations and that these determinants may be a widespread cause of copper resistance among the enterobacteria.

ACKNOWLEDGMENTS

This work was supported by Australian Research Council grant A09030665 and by U.K. Agricultural and Food Research Council grant AG6/537. The Royal Society and the British Council supported travel between our laboratories.

We thank S. Silver for a critical review of the manuscript.

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