SHORT COMMUNICATION

Use of a Synthetic Oligonucleotide to Identify a Chromosomal Gene for Chloramphenicol Acetyltransferase in a Plasmid-bearing *Flavobacterium*

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A micro-organism previously designated *Flavobacterium* sp. CB60 is resistant to chloramphenicol as a consequence of antibiotic acetylation by the enzyme chloramphenicol acetyltransferase and subsequent degradation of the acetylated product by co-metabolism. Although a 15.6 kb plasmid (pCB60) was demonstrated in this *Flavobacterium* strain, it did not appear to play a role in chloramphenicol acetylation. DNA hybridization was used to identify a fragment of DNA presumptively carrying the *cat* gene. The sequence of the synthetic probe was based upon known nucleotide sequences corresponding to the highly-conserved active site region of several chloramphenicol acetyltransferase variants. The structural gene for the enzyme in strain CB60 appeared to be chromosomal since the radioactive probe hybridized with a unique restriction fragment from chromosomal DNA but failed to do so with the DNA of plasmid pCB60.

INTRODUCTION

Chloramphenicol resistance (Cm^r) has been observed in many genera of bacteria, wherein resistance to chloramphenicol of more than $25 \mu\text{g ml}^{-1}$ is most commonly plasmid-borne and the result of antibiotic acetylation by the enzyme chloramphenicol acetyltransferase (CAT: EC 2.3.1.28) (Shaw, 1967; Suzuki & Okamoto, 1967). The genetic determinant (*cat*) is associated in Gram-negative bacteria with R-plasmids which can be assigned to a number of distinct incompatibility groups (Gaffney *et al.*, 1978). *Flavobacterium* sp. CB60, a Gram-negative isolate which is highly resistant to chloramphenicol, acetylates the antibiotic by means of the constitutive synthesis of CAT and further degrades the acetylated product in co-metabolism (Sussmuth *et al.*, 1979; Beschle *et al.*, 1982). Since a previous report implicated R-plasmids as vectors of resistance to ampicillin and erythromycin in clinical isolates of *Flavobacterium odoratum* (Kono *et al.*, 1980), it seemed appropriate to characterize the Cm^r determinant of *Flavobacterium* sp. CB60.

METHODS

Bacteria. The isolation and properties of *Flavobacterium* sp. CB60 have been described previously (Sussmuth *et al.*, 1979). *Flavobacterium* strains CB61 and CB67 are yellow pigmented flavobacteria which are chloramphenicol sensitive (Cm^s) and were kindly provided as controls by R. Sussmuth (Institute of Microbiology, University of Hohenheim, Stuttgart, FRG). *Methylophilus methylotrophus* (*mmeA*) was the gift of C. Boyd (Dept. of Genetics, University of Glasgow, UK) and *Escherichia coli* strains JM83 (*ara Δlac-pro strA thi φ80 dlacZΔm15*) and BW162 (*thr leu*) were obtained from G. Sharpe (ICI, Runcorn, UK). *Pseudomonas aeruginosa* PAO 1162 (*leu-38*) and PAO 1161 (*leu-38*) are both phenotypically recombination deficient (Rec^-) and modification proficient (Mod^+) as is *Pseudomonas putida* mt-2 KT 2440. These strains have been described by Bagdasarian *et al.* (1981) and were kindly provided by D. Jeenes (University College of North Wales, Bangor, UK).

Abbreviation: CAT, chloramphenicol acetyltransferase.

Plasmids. pSW 1.1 (Su^r Tc^r Mob⁺) and pUC 13 (Ap^r) were from G. Sharpe (ICI, Runcorn, UK). The use of pSW 1.1 was through the courtesy of W. Schuch (ICI, Runcorn, UK). Plasmid pJK111 (*cat*^{III}) encodes the type III enteric variant of CAT specified by plasmid R387; pSES9 carries the *cat*^c gene of staphylococcal plasmid pC221 (Shaw, 1983).

Media and growth conditions. Flavobacteria were grown according to Sussmuth *et al.* (1979). *E. coli* strains were grown on Luria medium or minimal medium with appropriate supplements and drugs as described by Maniatis *et al.* (1982). *M. methylotrophus* was cultivated in methanol-minimal medium (Windass *et al.*, 1980). *Pseudomonas* strains were grown as described by Timmis *et al.* (1978).

Plasmid preparations. Plasmids were prepared by the method of Birnboim & Doly (1979) using the following modifications in large scale preparations. After potassium acetate precipitation, the supernatants were shaken with aqueous phenol, precipitated with isopropanol and centrifuged to equilibrium in a caesium chloride buoyant density gradient containing ethidium bromide.

Chromosomal DNA preparations. Flavobacterial DNA was purified by the method of Chow *et al.* (1977) with the addition of a final caesium chloride buoyant density centrifugation step.

Transformation. *E. coli* strains were transformed by the method of Kushner (1978). The *Pseudomonas* transformation system was that of Bagdasarian & Timmis (1981).

Bacterial conjugations. Strains were conjugated by plate-mating as described by Barth *et al.* (1981).

DNA digestions and agarose gel electrophoresis. DNA samples were digested with commercial restriction endonucleases, using the manufacturers' recommended buffers, and analysed in 0.7% (w/v) agarose in Tris/acetate buffer (pH 8.1) as previously described by Maniatis *et al.* (1982). Molecular weights of DNA fragments were estimated from the migration of bacteriophage λ DNA fragments of known size.

Hybridizations. DNA fragments were transferred to nitrocellulose filters by the method of Southern (1975). Samples of the synthetic oligodeoxyribonucleotide 5'-CCATCACAGACGGCATGATG-3' (synthesized by Celltech, Slough, by the method of Patel *et al.*, 1982) were labelled using [γ -³²P]ATP and polynucleotide kinase by the method of Maxam & Gilbert (1980). Other DNA samples were nick-translated as described by Rigby *et al.* (1977). Filters were prehybridized for 4 h at 30 °C and probed with [³²P]oligonucleotide (sp. act. 5.5×10^7 d.p.m. μg^{-1}) for approximately 18 h. Filters were routinely washed in $2 \times \text{SSC}$ containing 0.5% SDS at 30 °C or at 37 °C for higher stringency in selected cases ($1 \times \text{SSC}$ is 0.15 M-NaCl/0.015 M-trisodium citrate, pH 7).

Ligation procedures and enzyme assays. DNA fragments were ligated to vectors using DNA ligase following treatment of linearized vector DNA with alkaline phosphatase followed by phenol extraction and ethanol precipitation. CAT was measured in crude extracts by the spectrophotometric and radioactive methods described by Shaw (1975).

RESULTS AND DISCUSSION

Presence of a 15.6 kb plasmid in *Flavobacterium* sp. CB60. Three very closely related *Flavobacterium* strains, CB60 (Cm^r), CB61 (Cm^s) and CB67 (Cm^s), were screened for the presence of plasmids after lysis and electrophoresis in agarose. Only strain CB60 was observed to contain a plasmid, the molecular weight of which was estimated to be approximately 15.6 kb by addition of the apparent sizes of fragments produced by digestion with individual restriction endonucleases. A map of the plasmid (designated pCB60) derived from the results of single and double digestions with restriction endonucleases *Pvu*II, *Eco*RI, *Sal*I and *Acc*I is shown in Fig. 1. The possibility that the Cm^r determinant was encoded by pCB60 was investigated both by attempting to transfer it to *Flavobacterium* strains CB61 and CB67, *E. coli*, *Pseudomonas* spp. and *M. methylotrophus*, and by probing with an active site synthetic oligonucleotide.

Transfer studies. The transformation with pCB60 of Cm^s *Flavobacterium* strains (CB61 and CB67), using conventional protocols for the transformation of *E. coli* and *Pseudomonas*, failed to yield Cm^r flavobacteria. All the *Pseudomonas* strains proved unsuitable because of their intrinsic resistance to chloramphenicol and low-level CAT activity. An effort was made to transform *E. coli* but no Cm^r ($10 \mu\text{g ml}^{-1}$) colonies were detected. Because of the absence of a transformation system for *M. methylotrophus* special conjugation vectors containing pCB60, pUC13, and the broad host range vector pWS1.1 were prepared by ligating the products of restriction endonuclease partial digests of each. Although such vectors were shown to replicate in *E. coli*, no consistent evidence of their transfer to *M. methylotrophus* was obtained (data not shown).

A synthetic oligonucleotide to identify CAT genes. The sequence around the active site of several different CAT genes from Gram negative and Gram-positive bacteria is highly conserved

Fig. 1. Restriction map of plasmid pCB60 using restriction endonucleases *AccI*, *EcoRI*, *PvuII* and *SalI*.

Fig. 2

Fig. 2. (a) 1.0% agarose gel showing banding pattern after ethidium bromide staining of: A, pBR322 digested with *TaqI* (negative control); B, pBR328 digested with *TaqI* (*cat*^I); C, pCB60 digested with *PstI*; D, pSES9 digested with *Sau3* (*cat*^R); E, pJK111 digested with *Sau3A* (*cat*^{III}). Arrows indicate the known position of the *cat* sequence in each case. (b) Autoradiograph of the DNA shown in (a) after transfer to nitrocellulose, by the method of Southern, and hybridization with [5'-³²P]oligonucleotide (see Methods). Hybridization only occurred with the bands known to contain the *cat* genes (arrowed).

Fig. 3

Fig. 3. Autoradiograph from a Southern transfer experiment in which chromosomal DNA from CB67 (A), CB61 (B), and CB60 (C) was subjected to digestion by endonuclease *PstI*, electrophoresis in 0.7% agarose and hybridization to a [32P]oligonucleotide probe as in Fig. 2(b). The discrete radioactive band for DNA from CB60, indicated by the arrow, was not observed for CB67 and CB61. The agarose gel itself revealed no detectable banding pattern before or after endonuclease digestion (not shown).

(Shaw, 1983). A 20-base synthetic oligonucleotide, complementary to the coding strand of the active site region of the gene for a type I CAT (*cat*^I, carried for example, by Tn9, pBR325, and pACYC184), was used in an attempt to identify novel as well as known *cat* elements by DNA:DNA hybridization. Initially, pBR322 (negative control), pCB60, pBR328 (*cat*^I), pJK111 (*cat*^{III}) and pSES9 (*cat*^R) were cut with appropriate restriction endonucleases, subjected to electrophoresis in 0.7% (w/v) agarose, transferred to nitrocellulose paper, and probed with [32P]oligonucleotide under high and low stringency conditions as described in Methods.

Autoradiography revealed that hybridization occurred only with plasmids carrying the three well-characterized genes specifying CAT. Both pCB60 and pBR322 failed to show any hybridization (see Fig. 2a, b), a result compatible with the view that pCB60 does not carry a typical gene for CAT. The alternative hypothesis that *Flavobacterium* sp. CB60 harbours *cat* on its chromosome was explored in similar fashion. Endonuclease *PstI* was used to digest pCB60 as well as total DNA from *Flavobacterium* strains CB60, CB61, and CB67. The digested DNA was

subjected to electrophoresis and the high stringency hybridization protocol as described; only the DNA of *Flavobacterium* sp. CB60 hybridized with the [³²P]oligonucleotide (Fig. 3). This result, and the failure of pCB60 to give a hybridization signal, strongly suggests that: (a) the *cat* gene in *Flavobacterium* sp. CB60 is chromosomal; (b) there is a substantial degree of homology at the active site between the CAT of strain CB60 and CAT variants studied previously; (c) the finding of pCB60 in this Cm^r strain was either fortuitous or reflects a secondary role which it may play in specifying gene products necessary for the subsequent metabolism of acetylated chloramphenicol (Beschle *et al.*, 1982). The studies reported here also emphasize the likely general utility of synthetic oligonucleotides as probes for prokaryotic genes which may not be expressed in bacterial hosts commonly used for cloning studies.

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