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Mechanism of Transferable Resistance to Chloramphenicol in *Haemophilus parainfluenzae*

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A clinical isolate of *Haemophilus parainfluenzae* resistant to chloramphenicol and tetracycline transferred both *cam* and *tet* determinants to *Escherichia coli* K-12 during mixed cultivation on solid media irrespective of the selection employed. The doubly resistant transconjugant was found to contain levels of the enzyme chloramphenicol acetyltransferase (CAT) comparable to those found in R plasmid-bearing chloramphenicol-resistant enteric bacteria. Purification of CAT from the transconjugant was achieved by affinity chromatography, and the electrophoretically homogeneous protein was compared with previously characterized CAT variants specified by R plasmids. Although the CAT associated with *cam* from *H. parainfluenzae* was found to be distinct from the three types described previously, its N-terminal peptide amino acid sequence was identical with that determined for a type II CAT. Attempts to demonstrate covalently closed circular deoxyribonucleic acid in the *H. parainfluenzae* donor and the *E. coli* transconjugant were unsuccessful. The *cam* and *tet* determinants were nontransmissible from *E. coli* but could be cotransferred following the introduction of a suitable conjugative plasmid.

Resistance of *Haemophilus* sp. to antibiotics has not been a clinical problem until recently. There is now reason to believe that plasmid-mediated transferable drug resistance is established in *Haemophilus influenzae* and *H. parainfluenzae* (1-6). Because of the importance of ampicillin in the management of *H. influenzae* meningitis, special efforts have been made to characterize the biochemical mechanism of resistance and to clarify the genetic and molecular events involved in the intergeneric transfer of the gene for a β-lactamase commonly found to be associated with R plasmids of the *Enterobacteriaceae* (4, 5). Chloramphenicol has been an exceedingly useful antibiotic for the treatment of serious *Haemophilus* infections in the past and is now of special value in the management of ampicillin-resistant cases. Reports of chloramphenicol-resistant *Haemophilus* have been few in number but may be of great importance as an index of future clinical problems (1, 7).

We report on certain aspects of chloramphenicol resistance in an isolate of *H. parainfluenzae* wherein the biochemical mechanism is acetylation of the antibiotic. However, the reaction is catalyzed by an enzyme which differs in a number of important respects from the most common variant of chloramphenicol acetyltransferase (CAT) specified by F-like plasmids but which is closely related to a CAT variant previously shown to be associated with a number of plasmids outside the F group. Although the evidence available is compatible with the view that the genetic loci for both chloramphenicol and tetracycline resistance in this strain are located on a plasmid, we have not been able to demonstrate the presence of plasmid DNA in either the original isolate of *H. parainfluenzae* or the transconjugant doubly resistant strain of *Escherichia coli* K-12 isolated after cocultivation of donor and recipient on solid media.

MATERIALS AND METHODS

Origin of the strains. The strain studied was isolated from the throat of a 1-year-old child at the St. Joseph Hospital in Paris. It was judged to be a typical isolate of *H. parainfluenzae* on the basis of its requirement for V factor (nicotinamide adenine dinucleotide) but not X factor (hemin) and by other standard biochemical studies (6).

Antibiotic susceptibility. The donor and recipient strains were tested with antibiotic disks (Institut Pasteur), and resistance was confirmed by the determination of minimal inhibitory concentration on solid media containing serial concentrations of antibiotics. When tested on chocolate agar containing IsoVitaleX (D.B. Merieux, France), the minimum inhibitory concentration of the *H. parainfluenzae* isolate was found to be 16 to 32 μg/ml for both chloramphenicol and tetracycline, in contrast to values of 0.5 to 1.0 μg/ml for susceptible control strains of the same species. The resistant strain was as susceptible as the control strain

to ampicillin, sulfonamides, streptomycin, and gentamicin.

Transfer of resistance into *E. coli* K-12. No transfer of resistance was found when the donor strain of *H. parainfluenzae* was grown in liquid medium with a potential *E. coli* K-12 recipient, strain J5, resistant to azide (*azi*) and rifampicin (*rif*). Transfer was observed, however, by the following technique. A chocolate agar-IsoVitaleX plate was inoculated with 5 ml of an 18-h culture of the doubly resistant *H. parainfluenzae*. After the excess growth medium was removed, the plate was incubated for 3 h at 37°C and then inoculated with 0.2 ml of a 1:100 dilution of an overnight culture of *E. coli* strain J5. After incubation for 18 h at 37°C, the plate was washed with sterile water, and aliquots were inoculated on unsupplemented nutrient agar plates containing sodium azide (250 μg/ml) plus either tetracycline (10 μg/ml) or chloramphenicol (7.5 μg/ml). After 48 h at 37°C, each plate contained 5 to 10 colonies of *E. coli* but none of *H. parainfluenzae*. All putative transconjugants were checked for and found to be resistant to azide, rifampicin, and both chloramphenicol (*cam*) and tetracycline (*tet*), irrespective of the selection made for resistance transfer. Strain J5 carrying the transferred *cam* and *tet* markers failed to transfer either after mixed cultivation in liquid or on solid media with *E. coli* C600 *nal*. However, after receipt of the conjugative plasmid RIP230(K) (3), strain J5 (*rif azi cam tet*) was able to transfer both *cam* and *tet* jointly to the *nal* recipient irrespective of the choice of donor markers used for selection.

Physical characterization of DNA. Attempts at demonstrating covalently closed circular (CCC) plasmid DNA by lysates of the donor *H. parainfluenzae* and in the transconjugant multiply resistant *E. coli* J5 were performed by equilibrium centrifugation in cesium chloride and ethidium bromide (2, 5). The results were unsuccessful, although control experiments performed under identical conditions successfully demonstrated CCC DNA in *E. coli* strains harboring well-characterized plasmids.

Preparation of cell-free extracts and purification of CAT. Strain J5 (*azi rif cam tet*) was grown in liquid Difco antibiotic medium no. 2 to late exponential phase of growth, and the bacterial cells were harvested by centrifugation. The cells were suspended in 50 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.8) containing 0.1 mM 2-mercaptoethanol and were sonically disrupted. The clear amber cell-free extract obtained after high-speed centrifugation was used for the assay of CAT by the spectrophotometric method (9). The specific activity of CAT in the cell extracts was comparable (0.5 U/mg of protein) to that observed for most R plasmid-bearing chloramphenicol-resistant isolates of *E. coli* (9). The cell extract was purified to homogeneity as judged by disc gel electrophoresis using an affinity chromatography system. The matrix of choice was a highly substituted Sepharose support with the free base of chloramphenicol attached through a spacer of 6-aminohexanoic acid (12). Elution from the affinity support was accomplished at pH 7.8 (50 mM tris(hydroxymethyl)aminomethane-hydrochloride) with 0.3 M sodium chloride containing 5 mM chloramphenicol and 0.1 mM 2-mercaptoethanol. The elution of non-CAT proteins was accomplished as described previously by a prior washing of the column with the above buffer lacking only chloramphenicol.

Characterization of CAT. The synthesis of the enzyme in strain *E. coli* J5 (*azi rif tet cam*) was a constitutive property, since growth in the presence of chloramphenicol or 3-deoxychloramphenicol (11) failed to increase the level of the enzyme over that observed in the absence of potential inducers. The general methods used in characterizing variants of CAT have been described in detail elsewhere (8, 9, 12). The ease with which the *H. parainfluenzae*-related CAT could be eluted from chloramphenicol-substituted Sepharose (12) was reproducible and in marked contrast to the requirement (see Table 1) of molar salt for the type I variants. The differences in elution behavior and electrophoretic mobility between other CAT variants and that from *E. coli* J5 (*azi rif tet cam*) were confirmed by mixing experiments in which each variant eluted or migrated independently of the other. The determination of the N-terminal amino acid sequence of CAT variants was performed by Edman degradation of the proteins following their covalent attachment to derivatized porous glass beads (10).

RESULTS AND DISCUSSION

Biochemical basis of chloramphenicol resistance. There seems little doubt that the presence of CAT is responsible for the chloramphenicol resistance phenotype in the clinical isolate of *H. parainfluenzae* and in the *E. coli* transconjugant which received both the *tet* and *cam* markers after mixed cultivation on solid medium. Although no enzymology was performed on the clinical donor strain, the presence of the enzyme in the recipient is diagnostic of gene transfer since (i) *E. coli* strain J5 had no detectable CAT before the experiments and failed to yield chloramphenicol-resistant colonies in the absence of cocultivation, and (ii) there are no known examples of chromosomal mutations in *E. coli* to *cam* that are mediated by the synthesis of CAT (8). The direct demonstration of chloramphenicol acetylation in *H. parainfluenzae* has been described by Kattan (7) in a clinical isolate from England (1) with the same *cam tet* resistance pattern as the Paris isolate described in this paper.

Properties of the acetylating enzyme. The variant of CAT isolated from the *E. coli* J5 transconjugant has been compared with selected properties of well-characterized types previously associated with R factors in the *Enterobacteriaceae* (8, 9, 12). Table 1 summarizes the results of these studies, which indicate that the CAT specified by the gene transferred from *H. parainfluenzae* to *E. coli* strain J5 is not identical with those known to be specified by R plasmids. Although the enzyme in the transconjugant is

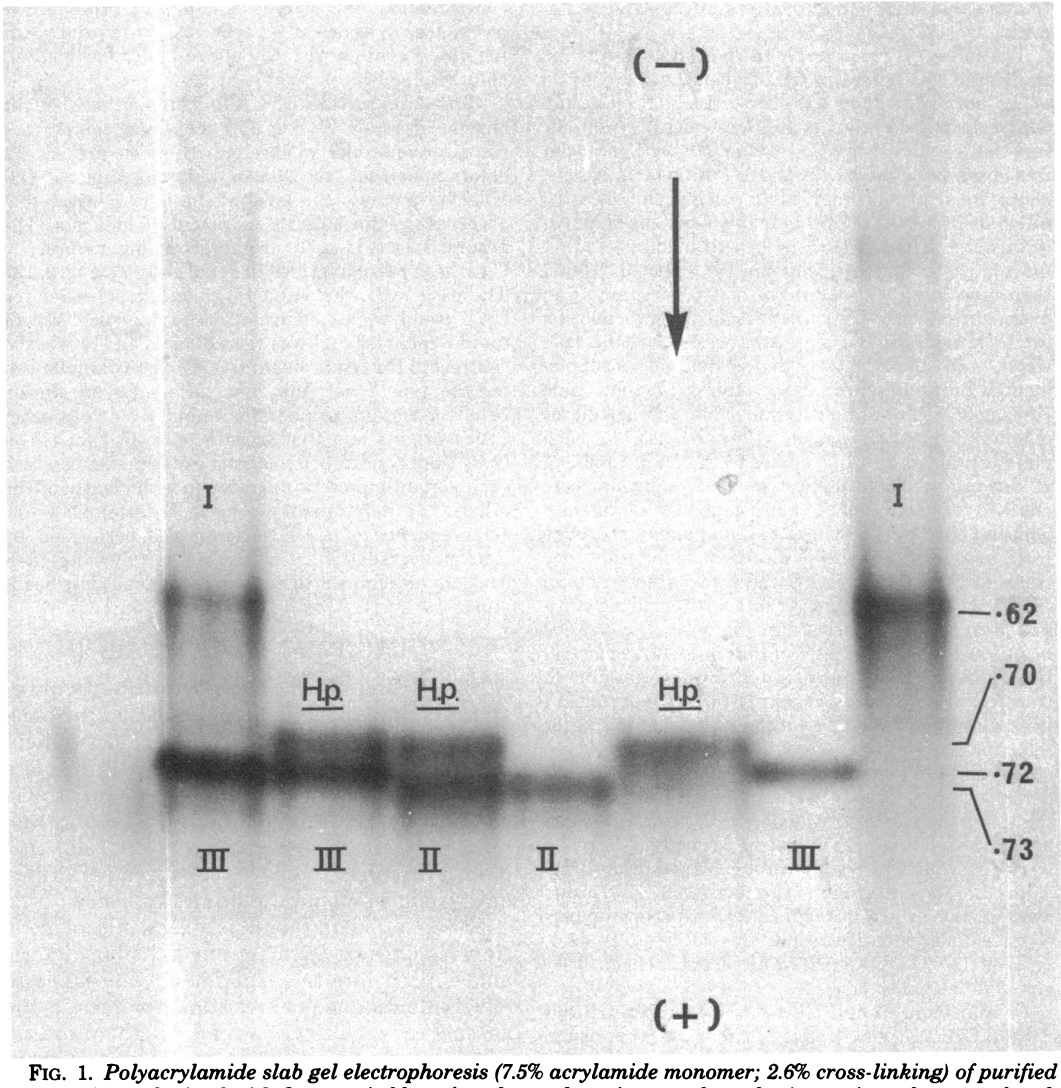

FIG. 1. *Polyacrylamide slab gel electrophoresis (7.5% acrylamide monomer; 2.6% cross-linking) of purified CAT variants. Stained with Coomassie blue after electrophoresis toward anode. Approximately 10 μg of each enzyme variant per sample. Mobilities in Table 1 were calculated from gel before drying. Symbols I, II, and III refer to CAT types specified by plasmids R429, S-a, and R387, respectively. H.p. refers to the CAT synthesized in the E. coli transconjugant after transfer of the chloramphenicol resistance determinant from H. parainfluenzae.*

superficially similar in certain respects to previously described variants of CAT, it differs in at least one important respect from each of the types expressed by R plasmids in *E. coli*. It is clearly distinguished from the common type I variant by differences in net charge and behavior on affinity chromatography, and from the type III variant on the basis of the higher subunit molecular weight of the latter. The observation that the transconjugant contains a 5,5′-dithiobis-(2-nitrobenzoic acid)-sensitive enzyme suggested that the CAT gene transferred from *H. parainfluenzae* might be related to that of the type II CAT variant in spite of differences in affinity chromatography behavior and net charge by electrophoresis. This suspicion was strengthened by the identity of the first seventeen residues of N-terminal amino acid sequence (Table 2).

We conclude that it is a novel class or type of CAT and that its presence in *H. parainfluenzae* can be explained neither by transfer of an R plasmid in toto nor by the translocation of the *cam* determinant from a commonly occurring R plasmid to a plasmid in *H. parainfluenzae*. The

TABLE 1. *Comparison of CAT from E. coli J5 transconjugant with enzyme variants specified by R plasmids*

Enzyme type	Representative R plasmids	Mol wt CAT monomer[a]	Sensitivity to 0.01 mM DTNB[b]	Reaction with anti-type I serum[c]	Molar NaCl required for elution in affinity chromatography[d]	Electrophoretic mobility[e]
I	Most F-like R plasmids	22,500	No	Yes	1.0	0.62
II	S-a, RA3, RA4	22,500	Yes	No	0.6	0.73
III	R387, R799	24,500	No	No	0.3	0.72
H. parainfluenzae (this study)		22,500	Yes	No	0.3	0.70

[a] Determined by polyacrylamide (11.5% gel) electrophoresis in 0.1% sodium dodecyl sulfate using cytochrome *c*, myoglobin, chymotrypsinogen, and ovalbumin as standards.

[b] CAT variants scored as insensitive showed a rate of inactivation less than 10% of that seen with type II when 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was incubated with enzyme (1 mg/ml of protein in 50 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer at pH 7.9) at 37°C in the absence of substrates.

[c] Only type I variant of CAT gave positive neutralization and precipitation tests with goat antiserum prepared against purified type I enzyme.

[d] The molar concentration of salt which must be present in addition to substrate (chloramphenicol) to achieve complete elution of CAT bound to affinity supports as described previously (12).

[e] Data for native enzyme when run in 7.5% polyacrylamide gels at pH 8.5. Fraction of total distance migrated by the marker dye. Measurements made from the bottom of the stacking gel to the leading edge of the CAT band stained with Coomassie blue.

TABLE 2. *N-terminal amino acid sequence of CAT variants*[a]

Enzyme type	Residue no.[b]																			
	1				5					10					15					20
I (R429)	Met	Glu	Lys	Lys	Ile	Thr	Gly	Tyr	Thr	Thr	Val	Asp	Ile	Ser	Glu	Trp	His	Arg	Met	Ala
II (S-a)						Met	Asn	Phe	Thr	Arg	Ile	Asp	Leu	Asn	Thr	Trp	Asn			
III (R387)						Met	Asn	Tyr	Thr	Lys)	Phe	Asp	Val	?	Asn	Trp	Val			
H. parainfluenzae (this study)						Met	Asn	Phe	Thr	Arg	Ile	Asp	Leu	Asn	Thr	Trp	Asn	Arg		

[a] Residues determined by thin-layer chromatography of phenylthiohydantoin derivatives and confirmed by back hydrolysis to free amino acids for analysis. (Lys) means inferred by "blank" on solid-phase sequence, since coupling was via Lys residues (10). Positions marked (?) signify ambiguous results.

[b] Alignment shown is empirical but based on identical residues at positions 9, 12, and 16 residues with similar physicochemical properties at positions 8, 11, and 13.

situation is, therefore, quite different from that observed in the case of the *amp* gene specifying the TEM variant of β-lactamase in *H. influenzae*, where there is circumstantial evidence for transfer of the *amp* transposon from an R plasmid to a plasmid resident in the *Haemophilus* host (4).

Our disappointment in failing to demonstrate CCC DNA by standard techniques in either the *H. parainfluenzae* donor or the *E. coli* K-12 recipient has been compounded by similar negative results in other cases of transmissible drug resistance in *Haemophilus* (A. Dang Van and D. H. Bouanchaud, unpublished data). Experiments are in progress to determine whether this is a fundamental property of *Haemophilus* plasmids or simply an indication that special techniques will be required to demonstrate CCC DNA in such isolates.

Our failure to achieve subsequent transfer of the *cam* and *tet* markers from the *E. coli* J5 transconjugant to strain C600 without the assistance of a resident conjugative plasmid such as RPI230(K) suggests that the conjugation functions specified by the putative plasmid in *H. parainfluenzae* are either not expressed in *E. coli* or have been lost in the process of transfer of the *tet* and *cam* determinants. An alternative hypothesis that chromosomal integration of the transferred genes has occurred in the *E. coli* recipient has not been ruled out and might also provide a reasonable explanation of our failure to demonstrate either CCC DNA or transmissibility of *cam* and *tet* in the transconjugant.

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