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Characterization of Transferable Plasmids from *Shigella flexneri* 2a That Confer Resistance to Trimethoprim, Streptomycin, and Sulfonamides

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A set of plasmids conferring resistance to several antibiotics, including the combination of trimethoprim and sulfamethoxazole, has been isolated from *Escherichia coli* following conjugative cotransfer from a clinical isolate of *Shigella flexneri* 2a. One of the plasmids, pCN1, was shown by subcloning and DNA sequencing to carry a gene encoding a trimethoprim-insensitive dihydrofolate reductase identical to that found in *E. coli* transposon 7. This plasmid was also shown to confer resistance to both streptomycin and spectinomycin by production of an adenylyltransferase that inactivated the drugs and the gene encoding this enzyme has also been sequenced. A second plasmid from the set, pCN2, was shown to inactivate streptomycin by a phosphotransferase mechanism and also to confer resistance to sulfonamides. The third plasmid from the set could not be correlated with a drug-resistance phenotype, but does appear to play a crucial role in plasmid mobilization.

The spread of transmissible plasmids that confer resistance to multiple antibiotics contributes to the difficult problem of controlling infectious diseases. Within a few years of the introduction of trimethoprim as an antimicrobial agent in the late 1960s, bacterial strains were isolated that were resistant to the drug (Datta and Hedges, 1972; Fleming *et al.*, 1972). Nevertheless, the efficacy of the drug, particularly when used in combination with sulfamethoxazole, led to its establishment as the treatment of choice for certain bacterial infections, such as shigellosis (Barada and Guerrant, 1980). In 1980 clinical isolates of *Shigella* resistant to the combination of trimethoprim and sulfamethoxazole (SXT), as well as to a number of other antibiotics, were reported for the first time (Bannatyne *et al.*, 1980; Taylor *et al.*, 1980). The high level of resistance to SXT exhibited by these strains was consistent with a plasmid-mediated mechanism; the lack of previous information on R-factors encoding trimethoprim resistance in *Shigella* originally prompted us to investigate one such isolate in more detail.

In this report we present physical and functional characterization of three plasmids that were isolated following cotransfer from a clinical isolate of *Shigella flexneri* 2a to *Escherichia coli*, and which, together, confer resistance to several antibiotics, including trimethoprim and streptomycin. The enzymatic mechanisms responsible for the observed resistance to trimethoprim and streptomycin have been determined and the genes encoding these two resistance phenotypes have been subcloned from one of these plasmids and fully characterized by DNA sequence analysis. The Tp^R-gene was found to be identical to the dihydrofolate reductase gene carried by transposon 7 from *E. coli;* the Sm^R-gene showed a high degree of homology with the sequence reported recently for the *aad*A gene of plasmid R538-1 (Hollingshead and Vapnek, 1985), al-

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though significant differences were found, particularly in the 5′ regions of the two genes.

MATERIALS AND METHODS

Isolation of antibiotic-resistance strains. An SXT-resistant clinical isolate of *S. flexneri* 2a was isolated in 1979 at Texas Children's Hospital, Houston, Texas from a patient residing in Dubai, Saudia Arabia by R. M. Lampe and E. O. Mason (personal communication). Antibiotic susceptibility studies performed by the method of Bauer *et al.* (1966) demonstrated that the isolate was also resistant to ampicillin, chloramphenicol and tetracycline, but sensitive to amikacin, cephalothin, gentamycin, kanamycin, nalidixic acid, and tobramycin. The strain was examined for the presence of conjugative resistance plasmids by a modification of the method of Datta and Hedges (1972). Transfer of resistance phenotypes from *Shigella* to a nalidixic acid-resistant strain of *E. coli* (HHB7) was monitored by plating onto agar containing nalidixic acid with or without other antibiotics. No colonies appeared on agar plates containing 25 μg/ml of either ampicillin or chloramphenicol. Colonies arose on agar plates containing 8/160 μg/ml of trimethoprim/sulfamethoxazole at a frequency of 10^{-5} per *E. coli* recipient cell, which indicated the simultaneous transfer of resistance to both of these two compounds. Transconjugants were purified and characterized by standard methods (Lampe and Mason, unpublished results). Other *E. coli* K12 strains used in transformation experiments were HHB1 (prototrophic W3110) and HHB22 (F^- *hsd*M^+ *hsd*R^- *lac*Y *leu*B6 Δ*trp*E5 *rec*A1).

Plasmid isolation and analysis. Purified plasmid DNA was prepared from *E. coli* strains by a lysozyme–sodium dodecyl sulfate (SDS) lysis procedure (Ratzkin and Carbon, 1977) followed by equilibrium banding in a cesium chloride/ethidium bromide gradient (Clewell, 1972). In some cases the plasmid content of strains was checked by analysis of DNA isolated by scaling down the procedure and omitting the CsCl banding or by an alkaline extraction method (Birnboim and Doly, 1979). Restriction endonuclease digestion reactions were carried out as recommended by the supplier (New England Biolabs). Fragments were analyzed on 0.8% agarose slab gels in the presence of 1 μg/ml ethidium bromide. Transformations were carried out on $CaCl_2$-treated cells according to the procedure of Dagert and Ehrlich (1979) with various antibiotic selections as described in Table 1. Subcloning of restriction fragments carrying antibiotic-resistance genes was carried out by standard methods (Maniatis *et al.*, 1982) and DNA sequence analysis on end-labeled fragments was performed by the Maxam–Gilbert chemical degradation protocol (Maxam and Gilbert, 1980). Dot matrix analyses were performed by the method of Zweig (1984).

Isolation of aminoglycoside 3″-adenyltransferase [AAD(3″)]. Bacterial cells containing the plasmid pCN1-3 were grown to early stationary phase in minimal medium (M9 supplemented with 10 g/liter casamino acids, 0.004% tryptophan, and 0.002% thiamine). All of the following procedures were carried out at 4°C unless otherwise indicated. The cells were washed twice with Buffer A (10 mM Tris, 25 mM NaCl, pH 7.6). Cells were disrupted in the presence of Buffer B (10 mM Tris, 10 mM $MgCl_2$, 25 mM NH_4Cl, 0.6 mM 2-mercaptoethanol, pH 7.6) with a Branson model 185 sonicator, using a microtipped probe (maximum power setting) for 6 × 15-s pulses. Soluble protein was collected by centrifugation and fractionated by ammonium sulfate precipitation. The 45–90% salt cut was resuspended in buffer C (10 mM Tris, 1 mM EDTA, pH 7.6). After concentration and extensive dialysis, the extract was applied to a Synchropak AX 300 HPLC column (SynChrom, Inc.). Proteins were eluted at room temperature from the column in four steps: (1) Buffer D (100 mM KPO_4, 1 mM EDTA, pH 7.6), (2) buffer D plus 0.5 M KCl, (3) buffer D with a salt gradient from 0.5 M KCl to 1.0 M KCl, and (4) buffer D plus 1.0 M KCl. Enzyme activity eluted in a single peak at 0.6 M KCl. Fractions containing enzyme activity were pooled, concentrated and dialyzed against

TABLE 1

ASSIGNMENT OF TRIMETHOPRIM AND STREPTOMYCIN-RESISTANCE DETERMINANTS TO PLASMIDS BY TRANSFORMATION AND PLASMID SCREENING[a]

Expt[b]	Selection	Transformants per μg DNA	Number screened	pCN plasmids detected					
				1	2	1, 2	1, 3	2, 3	1, 2, 3
1	Tp^R	69	10	7	0	0	2	0	1
2	Tp^R	113	4	0	0	2	2	0	0
3	Sm^R	2813	18	0	14	0	0	4	0
4	Sm^R	91	10	6	0	0	4	0	0
5	Sm^R	77	2	2	0	0	0	0	0

[a] Purified preparations of plasmid DNA were used to transform recipient cells as described under Materials and Methods. The recipient strain used in Experiment 1 was HHB22, while the recipient in Experiments 2–5 was HHB1. Transformants were selected and purified on minimal agar plates containing either 10^{-5} trimethoprim (Tp) or 100 μg/ml streptomycin (Sm) and the plasmid content was characterized by analysis of DNA minipreps.

[b] For Experiments 1, 2, and 3, plasmid DNA used in the transformation was isolated from HHB 46 which carries pCN1, pCN2, and pCN3. For experiment 4, DNA was prepared from HHB 48 which was isolated as a transformant of HHB 22 in Experiment 1 and shown to carry pCN1 and pCN3. In Experiment 5, DNA from HHB 47, which was isolated in the first experiment and shown to carry only pCN1, was used.

buffer D. Concentration was by dialysis versus buffer D plus saturating polyethylene glycol.

The concentrate was applied to a Synchropak AX 1000 HPLC column (SynChrom, Inc.). Proteins were eluted at room temperature in three steps: (1) Buffer D, (2) buffer D with a salt gradient from 0 M KCl to 1.0 M KCl, and (3) buffer D plus 1.0 M KCl. Enzyme activity eluted in a single peak at 0.35 M KCl. Fractions containing enzyme activity were pooled, concentrated and dialyzed against 50 mM ammonium bicarbonate. The sample was kept at room temperature for the remainder of the purification. The extract was concentrated to near dryness (30 μl) using a speed vacuum centrifuge.

Proteins in the extract were further fractionated by preparative SDS–polyacrylamide gel electrophoresis (PAGE) using a stacking gel (Laemmli, 1970). The gel was lightly stained with Coomassie brilliant blue for 20 min. The protein migrating at a molecular weight of ca. 31 kDa was excised and electroeluted following the procedure of Hunkapiller *et al.* (1983). The sample was concentrated by speed vacuum to 50 μl. A small sample (5 μl) was subjected to analytical SDS–PAGE followed by colorimetric silver staining (Sammons *et al.*, 1981) to determine the integrity of the protein. The remaining sample of protein was subjected to amino-terminal amino acid analysis on an Applied Biosystems model 470 gas-phase protein sequencer.

Enzyme assays. Dihydrofolate reductase (*dhfr*) assays were carried out by a spectrophotometric method described by Baccanari *et al.* (1975). One unit of *dhfr* activity is defined as the amount of enzyme required to reduce 1 μmol of dihydrofolate/min (after correction for dihydrofolate-independent NADPH oxidation) based on a molar extinction coefficient of 12.3×10^3 at 340 nm. Enzyme preparations were assayed for streptomycin adenylylation or phosphorylation by a phosphocellulose filter assay, measuring transfer of label to streptomycin from α-[^{32}P]ATP or γ-[^{32}P]ATP, respectively (Haas and Dowding, 1975).

RESULTS

Characterization of antibiotic resistance factors transferred from S. flexneri to E. coli. One of the SXT-resistant transconjugants, HHB46, obtained from the bacterial mating experiment described under Materials and Methods was selected for further study. This

E. coli isolate was shown to be resistant not only to the combination of trimethoprim/sulfamethoxazole but, as expected, to either trimethoprim or another sulfa drug, sulfanilamide, alone. This recipient strain was also shown to have concomitantly acquired resistance to streptomycin and spectinomycin, two drugs that were not tested with the original *S. flexneri* isolate. Neither the ampicillin- nor chloramphenicol-resistant phenotypes noted in the *S. flexneri* donor were transferred to HHB46.

Since the transmission of antibiotic resistance between bacteria is generally mediated by extrachromosomal R-factors (Davies and Rownd, 1972), an experiment was carried out to examine the plasmid content of HHB46. As shown in Fig. 1, Lane B, agarose gel electrophoretic analysis of a plasmid DNA preparation showed six distinct bands. By the same techniques no plasmid DNA could be detected in the HHB7 recipient strain (data not shown). Treatment of the plasmid preparation from HHB48 with the restriction endonuclease *Bam*HI prior to electrophoresis reduced the amount of DNA in bands a and b, and led to the appearance of band c, corresponding to a DNA fragment larger than 23 kb (lane c). Digestion of the DNA with the restriction endonuclease *Kpn*I had the same effect (lane E).

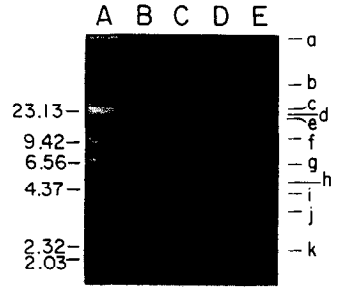

FIG. 1. Analysis of plasmids from HHB46 by agarose gel electrophoresis. A purified plasmid preparation from HHB46 was subjected to electrophoresis on a 0.8% agarose gel without further treatment (lane B) or after digestion with restriction endonuclease *Bam*HI (lane C), *Bgl*II (lane D), or *Kpn*I (lane E). The numbers on the left are sizes in kilobase pairs of λ *Hin*dIII fragments used as standards and the small letters on the right denote positions of ethidium bromide-stained bands referred to in the text.

Bands a and b also disappeared after digestion with *Bgl*II but, in contrast to the previous results, this treatment resulted in the appearance of two new high-molecular-weight bands (d and e, lane D); in this digest, bands h and k were also lost and a new DNA fragment (band i) with a size of about 4 kb appeared. In lane E it can also be seen that species f and j were cleaved with *Kpn*I which led to the appearance of band g with a size of approximately 6.3 kb. From these results, it was concluded that the initial plasmid preparation actually contained three distinct plasmids with sizes of >23 kb, 6.3 kb, and 4.1 kb (named pCN1, pCN2, and pCN3, respectively) with bands b, j, and k corresponding to the respective supercoiled conformations and bands a, f, and h corresponding to their open circular conformations generated during the isolation procedure. Thus bands c, g, and i represent full-length linear forms generated by restriction endonuclease cleavage of these plasmids. The presence of three plasmid species was subsequently verified by electron microscopy performed on the DNA preparation (data not shown); from this EM analysis, the size of pCN1 was more accurately determined to be about 45 kb.

Separation of plasmids by bacterial transformations. To determine the relationship between the pattern of antibiotic resistance expressed by HHB46 and the three plasmids present in the strain, a series of transformation experiments was carried out. Purified preparations of plasmid DNA were used to transform plasmid-deficient strains of *E. coli* K12 to trimethoprim or streptomycin resistance (Dagert and Ehrlich, 1979) and several transformants selected from each experiment were analyzed for plasmid content. The results are summarized in Table 1. The analysis of trimethoprim-resistant transformants selected in Experiments 1 and 2 revealed that pCN1 was sufficient to express that resistance phenotype since it was the only plasmid carried by each of the transformants; this conclusion was later verified by transformation of *E. coli* to trimethoprim resistance with a purified preparation of pCN1 alone (data not shown). The presence of pCN2 and/or pCN3 in several of

the strains from Experiments 1 and 2 was probably the result of unselected cotransformation. Examination of the ethidium bromide staining patterns for the electrophoretically separated plasmids from HHB46 (Fig. 1) shows that the concentrations of pCN2 and pCN3 were much higher than that of pCN1 in mixed plasmid preparations from that strain (presumably resulting from differences in plasmid copy number), which may have led to high cotransformation frequencies.

Analysis of the data presented for Experiment 3 in Table 1 led to the conclusion that the presence of pCN2 was sufficient for expression of a streptomycin-resistance phenotype, although cotransformation with pCN3 was noted in some cases. The frequency of transformation to streptomycin resistance in Experiment 3 was 25- to 40-fold higher than that for trimethoprim resistance in the first two experiments, which was attributed to the concentration differences between the two plasmids in the mixed population.

From the first three transformation experiments described in Table 1, a set of transformants was selected for further study. In testing for resistance against additional drugs, it was discovered that all of the strains were resistant to streptomycin, whether or not they carried pCN2. The results shown in Fig. 2 represent the plating efficiencies in the presence of varying concentrations of streptomycin for the plasmid-deficient strain (HHB1) and its plasmid-bearing derivatives generated as described in Table 1. Strains carrying pCN2 were found to be unaffected by high levels of streptomycin (200 μg/ml), whether or not pCN1 was present. Colony formation of HHB51, which carries only pCN1, was reduced at the drug concentrations tested although that strain was still much more resistant to streptomycin than was its plasmid-deficient parent. The higher level of resistance mediated by pCN2 may be a reflection of the specific gene it carries or of gene dosage since, as discussed earlier, pCN2 has a higher copy number than does pCN1. This suggestion that pCN1 also carries a gene specifying resistance to streptomycin was conclusively verified in Experiments 4 and 5 described in Table 1, where plasmid DNA from strains isolated in Experiment 1 was used to transform *E. coli* to streptomycin resistance. The antibiotic screening tests also led to the conclusions that resistance to spectinomycin was encoded by pCN1 and that the expression of sulfonamide resistance was dependent on the presence of pCN2. No antibiotic resistance phenotypes could be correlated with the presence of pCN3.

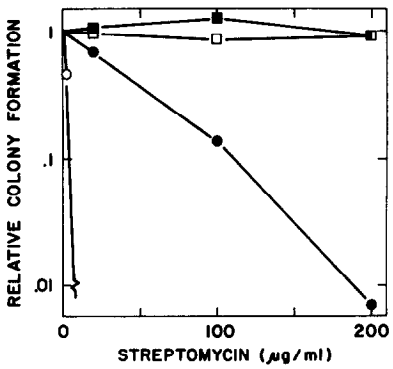

FIG. 2. Plating efficiency of *E. coli* strains in the presence of streptomycin. Relative colony formation on L-agar plates with varying concentrations of streptomycin were measured for the sensitive parental strain (○) and strains carrying pCN1 alone (●), pCN1 and pCN2 (□), or pCN2 and pCN3 (■).

Localization of resistance genes. Molecular subcloning was carried out to further localize the antibiotic resistance genes on pCN1 and pCN2 and to isolate them on fragments suitable for DNA sequence analysis. In the initial subcloning of pCN1, *Eco*RI-generated fragments were inserted into the *Eco*RI site of pBR313; both the Tp^R and Sm^R phenotypes were found to be encoded by a 12.5-kb segment carried by plasmid pCN1-1, shown in Fig. 3. A second round of subcloning, which involved insertion of fragments generated by partial digestion of pCN1-1 with *Hpa*II into the *Cla*I site of pBR322, gave recombinant pCN1-2, containing both the Sm^R and Tp^R genes on a 4.5-kb segment of DNA. Recloning of *Hpa*II fragments from this plasmid and subsequent restriction mapping localized the Tp^R gene to two contiguous *Hpa*II fragments of 350 to 550 bp in pCN1-7 (Fig. 3). The Sm^R

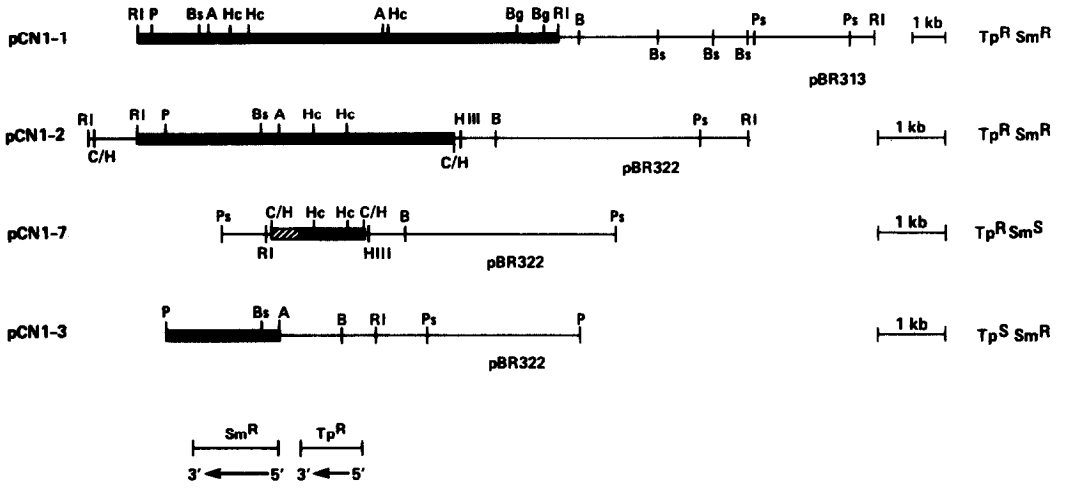

FIG. 3. Restriction maps of pCN1 subclones. The dark bars denote pCN1-derived sequences while the thin lines represent vector sequences. The positions of the Sm^R and Tp^R genes and the direction of transcription of the genes, based on DNA sequencing, is indicated at the bottom of the figure. Note that a different scale is used for pCN1-1. Abbreviations for restriction enzyme cleavage sites are the following: A = *Ava*I, B = *Bam*HI, Bg = *Bgl*II, Bs = *Bst*EII, C = *Cla*I, Hc = *Hin*cII, Hp = *Hpa*II, HIII = *Hin*dIII, P = *Pvu*II, Ps = *Pst*I, RI = *Eco*RI, C/H = junctions created by cloning *Hpa*II fragments into *Cla*I sites.

gene was localized to the 1.6-kb *Pvu*II–*Ava*I fragment of pCN1-2 by cloning this fragment into pBR322 to give recombinant pCN1-3 (Fig. 3). It was subsequently found that a Bal31-generated deletion derivative of pCN1-3 (not shown) that removed ca. 400 bp from the *Pvu*II end of the insert retained Sm resistance; insertion of exogenous DNA into the *Bst*EII site abolished the resistance phenotype. Thus the Tp and Sm resistance genes from pCN1 are tightly linked, although they can be expressed independently.

A number of subcloning experiments were also conducted to further localize the antibiotic resistance genes on pCN2 and the results are summarized in Fig. 4. Insertion of pBR322 at the unique *Eco*RI site did not disrupt either the Sm^R-gene or the Su^R-gene. Recloning of the two fragments generated by cleavage at the two *Pst*I sites into pBR322 gave a recombinant containing the larger of the two fragments that imparted Sm^R, but neither fragment alone was sufficient for expression of the Su^R-phenotype. The Sm^R-gene was subsequently localized to the region indicated in Fig. 4 by recloning fragments generated by partial digestion with *Hpa*II. The 1.1-kb *Ava*I fragment extending from 6.0 to 0.8 kb on the map was shown to encode sulfanilamide resistance and, thus, the Su^R-gene is located between the *Eco*RI site and *Ava*I site as indicated.

Mechanism of trimethoprim resistance. Plasmid-encoded resistance to trimethoprim has been previously associated with the production of novel forms of dihydrofolate reductase that are insensitive to this substrate analog (Amyes and Smith, 1974). In an attempt to identify such an enzyme in cells carrying pCN1, cell-free extracts were prepared

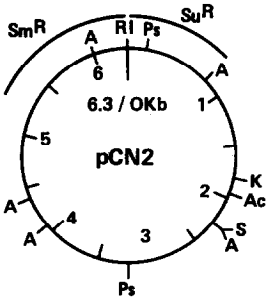

FIG. 4. Physical and functional map of pCN2. Abbreviations used for restriction enzyme recognition sites are as follows: A = *Ava*I, Ac = *Acc*I, K = *Kpn*I, P = *Pvu*II, Ps = *Pst*I, R = *Eco*RI, and S = *Sma*I.

from HHB22 and a derivative of that strain, HHB47, into which the plasmid pCN1 had been transferred by transformation. Following precipitation of nucleic acids with 1% streptomycin sulfate, the specific activities of dihydrofolate reductase in the preparations were 3.5 mU/mg protein from HHB22 and 8.0 mU/mg for HHB47 (see Materials and Methods), which indicated that an increased level of *dhfr* activity was associated with the presence of pCN1. The stability of *dhfr* activity in unfractionated cell extracts was also measured over an incubation period of 80 min at 45°C. No inactivation was observed for the *dhfr* activity from the host strain, HHB22. There was, however, a biphasic pattern of dihydrofolate reductase inactivation in extracts from HHB47. When the heat-treated material from HHB47 was assayed in the presence of 10 μM trimethoprim to eliminate the host activity, the plasmid-specified enzyme had a half-life of about 10 min and accounted for the rapidly inactivated component of the HHB47 preparation (data not shown).

For direct comparison of the plasmid-encoded *dhfr* with other characterized reductases, the complete nucleotide sequence of the pCN1-derived DNA fragments carried by the Tp^R subclone pCN1-7 (Fig. 3) was determined by the chemical degradation method (Maxam and Gilbert, 1980). Comparison with published sequences showed that this region is 100% homologous to the *dhfr* region of transposon 7 from *E. coli,* which encodes a polypeptide of 157 amino acid residues with a molecular weight of 17,600 (Fling and Richards, 1983; Simonsen *et al.,* 1983).

Mechanisms of streptomycin resistance. Two mechanisms have been described for plasmid-mediated resistance to streptomycin (Harwood and Smith, 1969; Ozanne *et al.,* 1969; Yamada *et al.,* 1968). These involve enzymatic adenylylation or phosphorylation of the drug using ATP as the second substrate in both cases. Only the enzyme possessing the former activity is capable of also inactivating spectinomycin. Since it was found earlier that pCN2 confers resistance to streptomycin and pCN1 confers resistance to both streptomycin and spectinomycin, strains containing those plasmids were assayed for both types of enzymes known to inactivate these aminoglycoside antibiotics. The enzymes were released from the cells by osmotic shock (Nossel and Heppel, 1966) and assayed as described by Haas and Dowding (1975). Enzyme preparations from HHB59 and HHB60 were used as controls; HHB59 carries a previously characterized plasmid (R12, Morris *et al.,* 1974) that encodes a streptomycin/spectinomycin adenylyltransferase (*aadA*) and HHB60 carries plasmid JR67 (Berg *et al.,* 1975) which has been shown to encode streptomycin phosphotransferase (*aphC*) (J. Davies, personal communication). The data presented in Fig. 5 show adenylyltransferase activity in extracts from HHB51 which carries only pCN1 and a phosphotransferase activity in extracts from HHB52 which carries only pCN2. Both activities were found to be expressed simultaneously in HHB46, which carries pCN1, 2, and 3. It was subsequently found that only about 8% of the total activity found in whole cell sonicates could be released by the osmotic shock procedure from cells producing the adenylyltransferase and thus, contrary to expec-

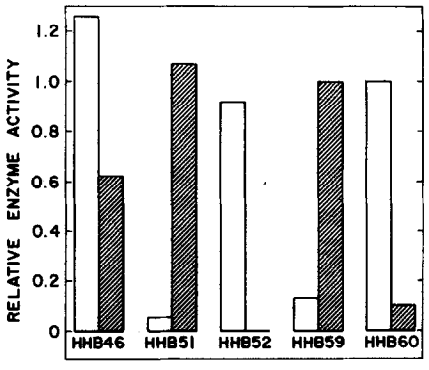

FIG. 5. Aminoglycoside antibiotic phosphotransferase (□) and adenylyltransferase (▨) activities in *E. coli* cell extracts. Relative enzyme activity (in arbitrary units per mg protein) was measured for strains containing pCN1, pCN2, and pCN3 (HHB46), pCN1 alone (HHB51), and pCN2 alone (HHB52). HHB59 and HHB60 are positive controls using extracts from cells containing previously characterized *E. coli* plasmids encoding adenylyltransferase or phosphotransferase activity, respectively.

tation, this activity is not predominantly localized in the periplasmic space.

The adenylyltransferase gene from pCN1 (referred to subsequently as *aad*A/pCN1) was sequenced by the strategy shown in Fig. 6. The nucleotide sequence with the predicted amino acid sequence for the protein is presented in Fig. 7. The amino terminus of the protein was established by amino acid sequence analysis on purified protein and, as shown, an appropriate ribosome binding sequence is found upstream from this position. The open reading frame predicts a protein with a molecular weight of 29,170, which is in reasonable agreement with estimates of protein size, as determined by SDS–PAGE (data not shown). As indicated in Fig. 7, long, nearly perfect direct repeats (50/55 nucleotides) were found immediately preceding and following the coding sequences; the significance of this highly unusual feature is unknown.

Comparison of the sequence shown in Fig. 7 with that of the *aad*A gene published by Hollingshead and Vapnek (1985) (referred to subsequently as *aad*A/R538-1) revealed extremely high conservation throughout most of the protein coding region. In fact, only three differences were found within the sequence from −11 to 827. At positions 684 and 747, a G and T, respectively, were determined for the *aad*A/pCN1 sequence, whereas an A and C were present at the corresponding positions in the *aad*A/R538-1 sequence; neither of these differences affects the predicted amino acid sequence. The R538-1 sequence also contains an extra GAA (Glu) codon between positions 705 and 706 that we did not find as demonstrated by the shift in the diagonal of the dot matrix shown in Fig. 8. Although the origin of this difference can only be surmised, it is possible that it was the result of an error in reading the former DNA sequence since there is some sequence redundance in this region. In contrast, the 5′ regions of the genes were completely different, as demonstrated by the dot matrix analysis shown in Fig. 8. The homology between the two genes is totally lost at 12 nucleotides preceding the protein start position indicated in Fig. 7, although Hollingshead and Vapnek proposed a protein start position from their sequence that lies an additional 168 bases 5′ of this site. It is possible that the two proteins are actually different, but, since our protein terminus was determined directly, we favor the interpretation that the additional extension of the open reading frame for the *aad*A/R538-1 gene was fortuitous and that, in fact, both proteins initiate at the same position. As shown by the matrix comparison (Fig. 8), sequence homology is also lost completely at about 60 bases following the TAA stop codons.

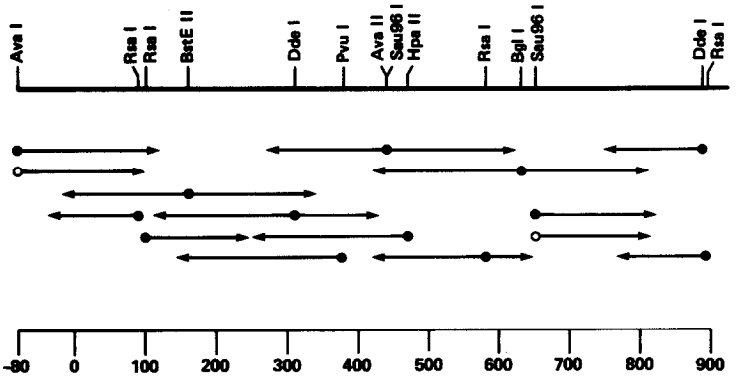

FIG. 6. A restriction endonuclease cleavage map and sequence analysis strategy for the aadA/pCN1 gene. DNA fragments isolated from plasmid pCN1-3 (see Fig. 3) were labeled at the 5′ end by using polynucleotide kinase (●) or at the 3′ end by using DNA Polymerase I (large fragment) (O). The direction and extent of the sequence determined for each fragment are indicated by the arrows. The numbering system corresponds to that used in Fig. 7.

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                                                                    oooo o
-72  CA GGATGACGCC TAACAATTCA TTCAAGCCGA CACCGCTTCG CGGCGCGGCT TAATTCAGGA GTTAAACATC

     Met Arg Glu Ala Val Ile Ala Glu Val Ser Thr Gln Leu Ser Glu Val Val Gly Val Ile
  1  ATG AGG GAA GCG GTG ATC GCC GAA GTA TCG ACT CAA CTA TCA GAG GTA GTT GGC GTC ATC

     Glu Arg His Leu Glu Pro Thr Leu Leu Ala Val His Leu Tyr Gly Ser Ala Val Asp Gly
 61  GAG CGC CAT CTC GAA CCG ACG TTG CTG GCC GTA CAT TTG TAC GGC TCC GCA GTG GAT GGC

     Gly Leu Lys Pro His Ser Asp Ile Asp Leu Leu Val Thr Val Thr Val Arg Leu Asp Glu
121  GGC CTG AAG CCA CAC AGT GAT ATT GAT TTG CTG GTT ACG GTG ACC GTA AGG CTT GAT GAA

     Thr Thr Arg Arg Ala Leu Ile Asn Asp Leu Leu Glu Thr Ser Ala Ser Pro Gly Glu Ser
181  ACA ACG CGG CGA GCT TTG ATC AAC GAC CTT TTG GAA ACT TCG GCT TCC CCT GGA GAG AGC

     Glu Ile Leu Arg Ala Val Glu Val Thr Ile Val Val His Asp Asp Ile Ile Pro Trp Arg
241  GAG ATT CTC CGC GCT GTA GAA GTC ACC ATT GTT GTG CAC GAC GAC ATC ATT CCG TGG CGT

     Tyr Pro Ala Lys Arg Glu Leu Gln Phe Gly Glu Trp Gln Arg Asn Asp Ile Leu Ala Gly
301  TAT CCA GCT AAG CGC GAA CTG CAA TTT GGA GAA TGG CAG CGC AAT GAC ATT CTT GCA GGT

     Ile Phe Glu Pro Ala Thr Ile Asp Ile Asp Leu Ala Ile Leu Leu Thr Lys Ala Arg Glu
361  ATC TTC GAG CCA GCC ACG ATC GAC ATT GAT CTG GCT ATC TTG CTG ACA AAA GCA AGA GAA

     His Ser Val Ala Leu Val Gly Pro Ala Ala Glu Glu Leu Phe Asp Pro Val Pro Glu Gln
421  CAT AGC GTT GCC TTG GTA GGT CCA GCG GCG GAG GAA CTC TTT GAT CCG GTT CCT GAA CAG

     Asp Leu Phe Glu Ala Leu Asn Glu Thr Leu Thr Leu Trp Asn Ser Pro Pro Asp Trp Ala
481  GAT CTA TTT GAG GCG CTA AAT GAA ACC TTA ACG CTA TGG AAC TCG CCG CCC GAC TGG GCT

     Gly Asp Glu Arg Asn Val Val Leu Thr Leu Ser Arg Ile Trp Tyr Ser Ala Val Thr Gly
541  GGC GAT GAG CGA AAT GTA GTG CTT ACG TTG TCC CGC ATT TGG TAC AGC GCA GTA ACC GGC

     Lys Ile Ala Pro Lys Asp Val Ala Ala Asp Trp Ala Met Glu Arg Leu Pro Ala Gln Tyr
601  AAA ATC GCG CCG AAG GAT GTC GCT GCC GAC TGG GCA ATG GAG CGC CTG CCG GCC CAG TAT

     Gln Pro Val Ile Leu Glu Ala Arg Gln Ala Tyr Leu Gly Gln Glu Asp Arg Leu Ala Ser
661  CAG CCC GTC ATA CTT GAA GCT AGG CAG GCT TAT CTT GGA CAA GAA GAT CGC TTG GCC TCG

     Arg Ala Asp Gln Leu Glu Glu Phe Val His Tyr Val Lys Gly Glu Ile Thr Lys Val Val
721  CGC GCA GAT CAG TTG GAA GAA TTT GTT CAC TAC GTG AAA GGC GAG ATC ACC AAG GTA GTC

     Gly Lys ***
781  GGC AAA TAA T GTCTAACAAT TCGTTCAAGC CGACGCCGCT TCGCGGCGCG GCTTAACTCA AGCGTTAGAG

851  AGCTGGGGAA GACTATGCGC GATCTGTTGA AGGTGG                                    886
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FIG. 7. Nucleotide sequence of the *aad*A/pCN1 gene. Nucleotide residue +1 denotes the A of the ATG used to encode the initiator methionine of the AAD(3″) protein, and residues preceding it are indicated by negative numbers. The amino acids predicted by the sequence are indicated. The first 10 amino acids were determined by sequencing the amino terminus of the isolated protein and are indicated by double underlining of nucleotides 1–30. The putative Shine–Dalgarno sequence is indicated with a (O) above each base. Homologous regions in the 5′- and 3′-untranslated regions are underlined.

DISCUSSION

In this paper we have described the analysis of a set of three plasmids transferred by conjugation to *E. coli* from an isolate of *S. flexneri* 2a that was resistant to a number of antibiotics, including the combination of trimethoprim and sulfamethoxazole. The largest of these, pCN1, has a size of approximately 45 kb and carries genes conferring resistance to trimethoprim and streptomycin; it also has been found to encode the genes required for its own transfer to recipient cells by conjugation (Roessler *et al.*, 1985). The second plasmid from the set, pCN2, is 6.3 kb in size and has been shown to carry genes conferring resistance to streptomycin and sulfonamides. Thus, selective pressure in the original transfer (SXT) was directed against one marker on pCN1 (Tp^R) and a second marker on pCN2 (Su^R). The third plasmid transferred, pCN3, has not been found to carry drug-resistance markers. However, it is interesting to note that subsequent experiments have shown that con-

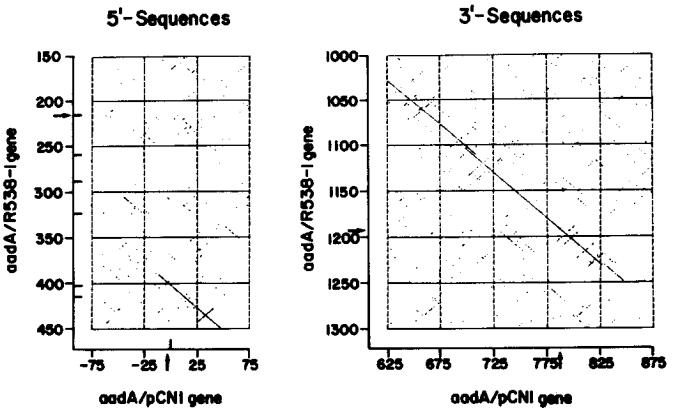

FIG. 8. Dot matrix comparison of the 5'- and 3'-sequences of the *aad*A/pCN1 and *aad*A/R538-1 genes. The nucleotide positions for the *aad*A/pCN1 gene are as shown in Fig. 7 and nucleotide positions for the *aad*A/R538-1 gene are according to Hollingshead and Vapnek (1985). Arrows indicate respective start and stop codons predicted for each gene. All in-frame start codons (both ATG and GTG) present in the 5'-sequence of the *aad*A/R538-1 gene are indicated along the inside of the vertical axis. A dot on the matrix represents a 9 for 10 match.

jugative transfer of pCN2 is dependent on the presence of pCN3 in the donor strain (Roessler *et al.*, 1985).

Brief reports have appeared of trimethoprim resistance in clinical isolates of *Shigella* from diverse locations, including Canada (Bannatyne *et al.*, 1980), Korea (Chun *et al.*, 1981), and Brazil (Tiemens *et al.*, 1984), but the mechanism of resistance was not examined in any of these cases. Resistance to trimethoprim has been previously shown in other species of bacteria to be mediated by R-factor genes encoding an altered form of dihydrofolate reductase that is insensitive to the compound (Amyes and Smith, 1974; Sköld and Widh, 1974); such genes are commonly carried on transposable genetic elements in close linkage with additional antibiotic resistance genes (Barth *et al.*, 1976; Shapiro and Sporn, 1977). The enzymes responsible for the plasmid-encoded resistance phenotype predominantly fall into two general classes on the basis of enzyme levels and degree of resistance to folate analogs (Pattishall *et al.*, 1977) and are clearly distinct from normal *E. coli dhfr*, which is a monomeric enzyme with a molecular weight of about 18,000. Although both of these types of R-factor encoded reductases have an apparent native molecular weight of about 36,000, they consist of either two (Type I) or four (Type II) identical subunits (Fling and Elwell, 1980; Novak *et al.*, 1983; Smith *et al.*, 1979). Examples of less common Tp^R-reductases with significantly different properties have also been reported (Fling *et al.*, 1982; Tennhammar-Ekman and Sköld, 1979). From preliminary characterization, it appeared that the *dhfr* encoded by the pCN1 plasmid from *S. flexneri* should be classified as a Type I enzyme; in fact, subsequent DNA sequencing revealed that it was identical to the prototypic Type I *dhfr* gene on Tn7 carried by the R483 plasmid. It has been noted previously (Fling and Richards, 1983; Simonsen *et al.*, 1983) that this reductase shows no significant homology with characterized Type II reductases from R67 (Stone and Smith, 1979) and R388 (Swift *et al.*, 1981; Zolg *et al.*, 1981) and only very limited homology with *E. coli dhfr* (Smith and Calvo, 1980) and, thus, the evolutionary relationship of these procaryotic reductases remains uncertain. Fling *et al.* (1982) have shown that a Tp^R-isolate of *Shigella sonnei* from Brazil (P570) gave hybridization only with a Type II-specific DNA probe. Therefore, the isolate that served as the basis for these studies appears to be the first documented example of the presence of Tn7 sequences in

Shigella and further expands the host range of this rapidly spreading element (Richards and Nugent, 1979).

We have also demonstrated that the two genes imparting streptomycin resistance that were localized to pCN1 and pCN2 code for two distinct enzymes. The gene on the conjugative plasmid pCN1 encodes an enzyme that inactivates streptomycin and spectinomycin by adenylylation, while the gene on pCN2 produces an enzyme that inactivates only streptomycin by phosphorylation of the drug, as has generally been found to be the case with nonconjugative plasmids (Kawabe *et al.*, 1978). Plasmid genes coding for enzymes responsible for streptomycin resistance are frequently coupled with genes that confer sulfonamide resistance and, in fact, resistance to sulfonilamide was also shown to be conferred by a gene on pCN2. The enzymatic mechanism responsible for this resistance was not examined but other workers have shown that bacterial resistance to sulfonamides results from the production of drug-resistant forms of dihydropteroate synthase (Swedberg and Sköld, 1980, 1983). It appears that most, if not all, nonconjugative plasmids conferring streptomycin and sulfonamide resistance can be classified into two separate groups on the basis of molecular weights and replicative functions. Those related to the prototype R300B (Barth and Grinter, 1974; Grinter and Barth, 1976) are approximately 8.8 kb in size and are members of the incompatibility group IncQ, while plasmids similar to pBP1 (van Treeck *et al.*, 1981) are smaller (ca. 6.4 kb) and appear to be based on a different replicon, although the resistance genes still appear to be highly homologous (van Treeck *et al.*, 1981). Based on functional and physical properties, including comparison of partial restriction maps, pCN2 closely resembles several reported plasmids of the latter class including pBP1 (van Treeck *et al.*, 1981), pSAS1206 (Shareck *et al.*, 1983), and pGSO3B (Swedberg and Sköld, 1983). However, further analysis will be required to clarify the apparent close evolutionary relationship of these plasmids.

By a combination of DNA and protein sequencing, we have determined the complete amino acid sequence for the AAD(3″) activity responsible for the observed resistance to streptomycin and spectinomycin of cells containing the *aad*A/pCN1 gene. Although the results here are in disagreement with those of Hollingshead and Vapnek (1985), it is important to note that their assignment of a translation start site was not verified by protein sequencing, but was based on the assumption that the amino terminus of the protein should contain a hydrophobic signal peptide that would direct its localization into the periplasmic space. We have found that, in fact, most of the enzymatic activity is cytoplasmic and, in addition, the molecular weight of 29,170 predicted from our sequence is in much closer agreement to published estimates of the size of the mature AAD(3″) protein (Alton and Vapnek, 1979; Dempsey and McIntire, 1979; Hollingshead and Vapnek, 1985). Therefore, we feel that the sequence presented in this paper correctly represents that of the streptomycin/spectinomycin adenylyltransferase enzyme and, based on the close linkage of the gene with the Tp^R-dhfr gene described earlier, may be expected to be identical to the *aad*A gene of Tn7.

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