JOURNAL OF BACTERIOLOGY, Aug. 1991, p. 5105–5109
0021-9193/91/165105-05$02.00/0

Vol. 173, No. 16

Analysis of *Streptococcus pneumoniae* Sequences Cloned into *Escherichia coli*: Effect of Promoter Strength and Transcription Terminators

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Received 28 January 1991/Accepted 24 May 1991

Difficulties encountered in the cloning of DNA from *Streptococcus pneumoniae* and other AT-rich organisms into ColE1-type *Escherichia coli* vectors have been proposed to be due to the presence of a large number of strong promoter-acting sequences in the donor DNA. The use of transcription terminators has been advocated as a means of reducing instability resulting from disruption of plasmid replication caused by strong promoters. However, neither the existence of promoter-acting sequences of sufficient strength and number to explain the reported cloning difficulties nor their role as a source of instability has been proven. As a direct test of the "strong promoter" hypothesis, we cloned random fragments from *S. pneumoniae* into an *E. coli* vector containing transcription terminators, identified strong promoter-acting sequences, and subsequently removed the transcription terminators. We observed that terminator removal resulted in reduced copy numbers for the strongest promoter-acting sequences but not in reduced promoter strengths or altered plasmid stabilities. Our results indicate that promoters strong enough to require transcription terminators for plasmid stability are probably rare in *S. pneumoniae* DNA.

Attempts to clone DNA from *Streptococcus pneumoniae* into ColE1-type multicopy plasmids of *Escherichia coli* have often met with significant difficulty (8, 23, 24, 29, 32, 35). It has been proposed that this difficulty is related either to the production of pneumococcal proteins that are detrimental to *E. coli* or to the presence of large numbers of random sequences with strong promoter activity that result in instability in *E. coli*. The former explanation accounts for the inability to clone *amiA* and *malX* in the absence of either mutations in the cloned sequences or reductions in plasmid copy number (23, 35). However, certain *S. pneumoniae* sequences, e.g., those containing the genes *com*, *hexA*, *hexB*, and *recP*, have proven unstable in ColE1-type multicopy vectors in *E. coli* but have been cloned by utilizing plasmids of gram-positive bacteria in *S. pneumoniae* or by using ColE1-type multicopy vectors containing transcription termination sequences for cloning in *E. coli* (8, 24, 29, 32). Vectors containing transcription terminators have been reported to increase the efficiency of shotgun cloning of *S. pneumoniae* DNA and to increase the number of random, stable clones obtained, compared with an identical vector lacking terminators (9).

Because *S. pneumoniae* DNA is 61% AT (22), the likelihood of cloning random sequences that resemble the *E. coli* σ^{70} promoter sequence is high (28). To recognize actual promoters, *S. pneumoniae* RNA polymerase is expected to have a more stringent consensus sequence requirement than does *E. coli* RNA polymerase (26, 27). Indeed, it has been shown that more strong promoter-acting sequences are obtained with random *S. pneumoniae* DNA fragments than with random *E. coli* fragments (9). In addition, we have observed that *S. pneumoniae* sequences that function as promoters in *E. coli* do not necessarily do so in *S. pneumoniae* (38). Thus, nonpromoter sequences of *S. pneumoniae* may act as promoters in *E. coli*, and actual promoters may have increased strength in *E. coli*. Like the very strong promoters from *E. coli* bacteriophages and from ribosomal operons, these sequences may destabilize normal *E. coli* vectors as a result of excessive transcription that interferes with normal plasmid replication functions (9, 27, 35, 36). Such strong promoters have been cloned in vectors containing transcription termination sequences that prevent readthrough into the plasmid origin (4, 6, 13).

The observed difficulties in cloning numerous *S. pneumoniae* chromosomal regions and the results obtained by using transcription termination vectors suggest that features of the DNA sequence may contribute to instability. Here, we describe experiments designed to directly test the "strong promoter" hypothesis.

MATERIALS AND METHODS

Bacterial strains. *S. pneumoniae* D39 (1) was grown at 37°C in Todd-Hewitt broth supplemented with 0.5% yeast extract (Difco Laboratories, Detroit, Mich.). *E. coli* DH1 (*supE44 hsdR17 recA1 gyrA96 thi-1 relA1*) (15) was grown at 37°C in L broth or on L agar (25). Ampicillin (AP) and chloramphenicol (CM) were added to final concentrations of 50 and 25 μg/ml, respectively, unless otherwise noted.

Construction of clones. Chromosomal (16) and plasmid (2) DNAs were isolated and purified (30) by using published techniques. Standard techniques were used for *E. coli* transformations (11), plasmid size screenings (17), and DNA digestions and ligations. Specific fragments for cloning were excised from 0.5% agarose gels and purified by using GeneClean (Bio 101, La Jolla, Calif.). Confirmation of the expected clone construction was obtained by restriction mapping.

Transcription terminators downstream of the *cat* gene were removed from pKK232-8 (to yield pJD1002), or from clones in the pKK232-8 library, by deleting the *Fdi*II fragment containing these sequences. For these constructions, the appropriate fragment of a partial *Fdi*II digest was gel

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purified, religated, and transformed to *E. coli*. Alternatively, inserts originally obtained in pKK232-8 were subcloned to pJD1002 as *Pst*I or *Pvu*I-*Sal*I fragments.

Terminators upstream of the *cat* gene were removed by digestion with *Sma*I followed by partial digestion with *Ssp*I. The desired gel-purified fragment was religated and transformed to *E. coli*.

Cloning of known promoters. The Tn*9* promoter removed in the construction of pKK232-8 (4) was isolated from pVA891 (21) as a 426-bp *Pvu*II fragment and inserted into the *Sma*I site of pKK232-8. The *tac* promoter was subcloned into the *Bam*HI site of pKK232-8 from pKK223-3 (12) as a 268-bp *Bam*HI fragment. The λp_L promoter was isolated from pRE1 (31) as a 377-bp *Mro*I-*Fdi*II fragment, which was blunt ended with the Klenow fragment of *E. coli* DNA polymerase I, and inserted into the *Sma*I site of pKK232-8. The resulting plasmids are pJD1001, pJD402, and pJD415, respectively.

Enzyme and protein assays. Mid-log-phase *E. coli* cells, grown in triplicate in L broth with AP (vector only) or with AP and CM (promoter-containing clones), were harvested by centrifugation, washed twice, resuspended in 100 mM Tris-HCl (pH 7.8), and then frozen at −70°C for at least 1 h. Thawed suspensions were sonicated three times for 10 s each and centrifuged, and the supernatant fluids were stored at −70°C. Extracts were measured for chloramphenicol acetyltransferase (CAT) (33) and β-lactamase (20) activities in triplicate and for total protein (3) in duplicate. Values were normalized to total protein content.

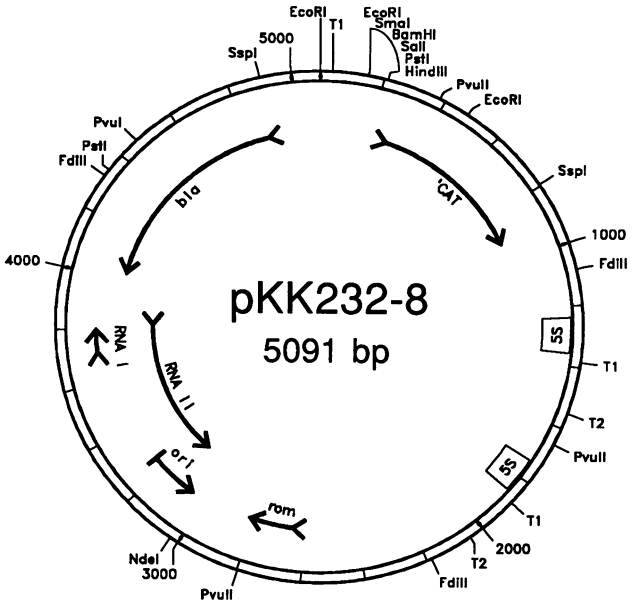

FIG. 1. Map of plasmid pKK232-8 showing the locations of restriction sites and the transcription terminators (T1 and T2). Genes encoding β-lactamase (bla), the promoterless CAT, and 5S rRNAs are indicated inside the circle, as are replication and replication control transcripts, replication primer RNA II, inhibitor RNA I, RNA one modulator protein (rom), and the replication origin (ori).

RESULTS AND DISCUSSION

Identification of promoter-acting sequences. As the first step in testing the strong promoter hypothesis, we cloned random *S. pneumoniae* DNA fragments into pKK232-8, a ColE1-type *E. coli* vector containing a promoterless *cat* gene flanked by strong trnascription terminators from the *E. coli* *rrnB* operon (Fig. 1) (4). In order to reduce the number of large fragments obtained and thus minimize the likelihood of cloning potentially lethal products, our library was constructed from a complete *Sau*3A digest of the pneumococcal chromosome ligated into the *Bam*HI site of pKK232-8. One set of clones containing promoter-acting sequences was identified by first selecting *E. coli* transformants on ampicillin-containing medium (Ap^r) and then screening for CM resistance (Cm^r). Of 74 random Ap^r transformants, 59 (80%) had inserts. Of these, 49 (83%) were Cm^r. By selecting directly on CM, an additional 21 isolates were obtained. As expected, the majority of the clones contained relatively small inserts (data not shown). Previously, resistances of 200 μg of CM or more per ml have been defined as being indicative of strong promoter activity (9, 32). Isolates from both of our libraries exhibited a wide range of resistance levels, from 25 to 1,600 μg of CM per ml. Most isolates were resistant to at least 200 μg of CM per ml, and several were resistant to 400 to 1,600 μg/ml. Isolates with levels of resistance of ≥800 μg of CM per ml were rare, making up only 5% of the library.

Promoter strengths, plasmid copy numbers, and plasmid maintenance. For further studies, 10 clones from the AP-selected library and two from the CM-selected library were arbitrarily chosen to represent the range of CM resistances observed with all isolates (Table 1). Digestion with *Sma*I and *Sal*I or with *Sau*3A alone revealed that each of the 12 clones contained a single, unique *Sau*3A fragment. Because levels of CM resistance or CAT activity alone may not accurately reflect promoter strengths, the level of CAT was normalized to the level of β-lactamase to yield the CAT/β-lactamase ratio, a final measure of promoter strength. Normalization to β-lactamase should control for changes in copy number that may result from insertion of a foreign fragment into the

TABLE 1. Characteristics of promoter-acting fragments cloned from *S. pneumoniae* and of defined *E. coli* promoters

Isolate	Size (bp)	Cm^r (μg/ml)[a]	Level of production of:		CAT/BLA[b]
			CAT[c]	BLA[d]	
1	300	25	1.3	1.9[e]	0.7
2	400	100	0.4	2.0	0.2
3	700	100	0.6	1.0	0.6
4	350	200	1.5	1.6	0.9
5	2,000	200	1.0	0.6[e]	1.8
6	1,900	200	0.9	0.5[e]	2.0
7	700	200	1.5	0.6[e]	2.6
8	1,300	200	2.0	0.7	3.0
9	1,100	400	1.5	0.5[e]	3.2
10	450	400	2.6	0.8	3.2
11	200	800	2.8	0.6	4.3
12	1,400	1,600	8.6	1.6	5.3
pKK232-8		<25	0.03	1.0	0.03
pJD1001 (*cat*)	426	800	10.0	1.2	8.5
pJD402 (*tac*)	268	800	8.9	1.8[e]	5.0
pJD415 (λp_L)	377	ND[f]	47.7	2.6[e]	18.2

[a] Maximum CM concentration to which isolate was resistant.
[b] The level of CAT was normalized to the level of β-lactamase to yield the CAT/β-lactamase ratio, a final measure of promoter strength.
[c] CAT production is expressed in units as calculated by Shaw (33).
[d] β-Lactamase (BLA) production is expressed relative to the level produced by the parent plasmid pKK232-8.
[e] Significantly different from values for control, pKK232-8, at $P \leq 0.05$.
[f] ND, not determined.

vector. The greatest promoter strengths observed with these 12 clones were close to that of the *E. coli tac* promoter, one of three well-characterized promoters that we cloned for comparison.

The insertions did not significantly alter maintenance of pKK232-8, as determined by the percentage of antibiotic-resistant CFU among cells grown in the absence of selection and by the growth rate in the presence and absence of selection (data not shown). Some insertions did affect plasmid copy number but not in a manner that correlated with promoter activity (Table 1). The decreased copy numbers observed with isolates 5, 6, 7, and 9 were insertion specific, as a normal copy number was observed when the insertions were removed, and subcloning of the insertions resulted in a reduced copy number of the new vector (data not shown). For isolates 1, 2, and 4, the increase in copy number was apparently due to plasmid amplification. Doubling times of these isolates were significantly longer in the presence of CM than in its absence. The copy numbers were normal when CM was omitted from the medium.

Effect of terminator removal. Certain strong promoters from *E. coli* bacteriophages and ribosomal operons are unstable in the absence of transcription terminators, i.e., either they are not clonable or they acquire deletions or other mutations when cloned (4, 13, 36). The mechanism of this type of plasmid instability is not fully understood. However, it is thought to be due to excessive transcription into the replication origin, leading to increases in Rom and RNA I and thereby decreasing the copy number (5, 6, 36). For plasmids that partition randomly, as do ColE1-type plasmids, a drastic reduction in copy number can lead to plasmid loss (37). To determine whether the transcription termination sequences in our pKK232-8 clones containing *S. pneumoniae* DNA were essential for plasmid stability, we either removed the terminators downstream of the *cat* gene or placed the promoter-acting fragments in pJD1002, an otherwise identical vector lacking these terminators. If the promoter strength were too great for stable maintenance, clones would be obtained in the unprotected vector only rarely, probably as the result of a promoter-down mutation. The removal of terminators should also identify clones in which the terminators had been increasing stability in some manner other than the prevention of read-through transcription. For example, by changing the topology of the plasmid, the hairpin structures of transcription terminators might reduce conformational changes associated with AT-rich DNA (34), which can cause deletions (14). However, we observed that when the terminators were removed, a large number of stable clones containing the expected fragment was obtained for each of the plasmids listed in Table 1, except λp_L. The latter observation confirmed that the defined instability was demonstrable in our system. The levels of CAT activity remained virtually unchanged for all of the clones except isolate 2, for which activity was increased, and isolates 7 and 8, for which activities were slightly decreased (Fig. 2A). In the absence of the downstream terminators, isolates 4, 9, 10, 11, and 12 and *tac* showed a reduction in plasmid copy number (Fig. 2B). Except for isolate 4, this group consists of the strongest promoter-acting pneumococcal fragments. Some disruption of replication may have occurred in these clones. However, in no case was copy number reduced to the extent that growth in the presence of AP selection was significantly affected. Thus, in the absence of downstream termination sequences, we observed no significant decreases in promoter strength except in the case of isolate 7 (Fig. 2C); all other significant changes were apparent increases (isolates 2, 9, and 12 and *tac*).

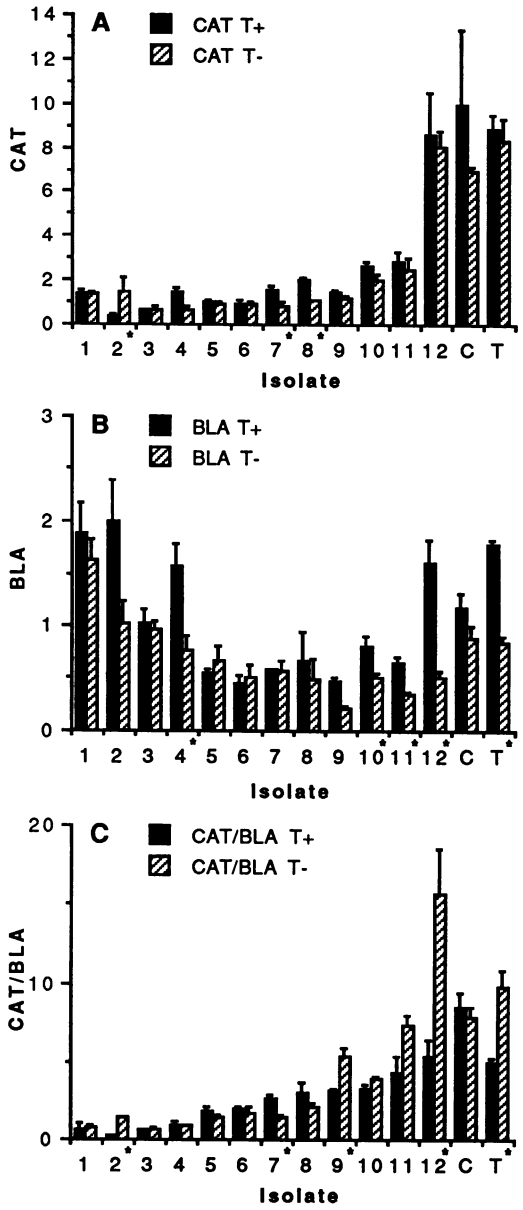

FIG. 2. Effect of downstream terminator removal on CAT production (A), plasmid copy number as measured by β-lactamase (BLA) production (B), and aparent promoter strength (C). β-Lactamase production is expressed relative to the level produced by the parent plasmid pKK232-8 or to that of pJD1002 for the presence (T+) and the absence (T−) of transcription terminators, respectively. C and T denote promoter comparison plasmids pJD1001 (*cat*) and pJD402 (*tac*), respectively. All values are geometric means; error bars represent standard errors. Asterisks indicate statistically significant differences ($P \leq 0.05$) between the T+ and T− values.

Instability could also result from excessive transcription into the origin from the opposite direction (6). Therefore, transcription terminators between the multicloning site and the *bla* gene were removed from several of the original isolates and from some of the derivatives lacking the downstream terminators. In no case were these terminators nec-

essary for stability, as all fragments were obtained in unprotected vectors at high frequency, and CAT and β-lactamase levels were unaffected (data not shown).

Conclusions. Our results indicate that although *E. coli*-like promoter sequences occur frequently in the *S. pneumoniae* chromosome, promoters of sufficient strength to cause instability in the absence of transcription terminators are probably rare. The experiments indicate that promoters as strong as that of λp_L, which is one of the strongest promoters known (18), would be expected to require terminators for stability in pKK232-8. However, we did not isolate any such promoters from *S. pneumoniae*. Although we cannot rule out the possibility that cloning vectors used by other investigators are more sensitive to promoter effects than is pKK232-8, all of the reported cloning difficulties have involved plasmids with ColE1-type origins, which could be expected to be affected by promoter activity in a manner similar to that of pKK232-8. Additionally, we observed no instability when one of our strongest promoter-acting fragments (from isolate 11) was cloned into pUC18 and pUC19 (data not shown).

Several explanations may account for the differences between our results and those suggesting that strong promoters represent a major source of cloning difficulty. First, vectors of unequal copy numbers, i.e., pUC19 and pJDC9, were used in the initial experiments, suggesting that termination vectors improved the yield of fragments larger than 3 kb but not of smaller fragments (9). The higher copy number of the terminator-minus vector pUC19 would tend to amplify the effects of detrimental products, which would be more likely to occur in larger fragments. Second, comparison experiments with pJDC9 and its terminator-minus derivative pJDC10 may have been affected by the presence of the potentially lethal *kil* gene product encoded by the vectors (7, 10, 19). Promoter-containing fragments cloned in pJDC10 could transcribe *kil* but would be prevented from doing so in pJDC9 by the interposed transcription terminator. Therefore, the efficiency of cloning promoters would be reduced in the terminator-minus vector because of the lethal product on the vector, not because of any effect of the promoter per se. Third, although we examined a number of clones, our experiments do not rule out the possibility that terminators may resolve problems unrelated to promoter strength, such as reductions in conformational changes that could result in deletions (14, 34). Finally, the majority of the reported problems have occurred in the initial stages of cloning, i.e., when larger fragments were being cloned. Thus, the observed problems could be related to multiple effects, including lethal products and DNA sequence-specific effects, as well as vector-specific problems.

ACKNOWLEDGMENTS

We thank David E. Briles for his support of this work and for assistance with statistical analysis of the data. We also thank Thomas Elliott for the kind gift of plasmids pKK223-3 and pRE1 and William H. Benjamin, Jr., for useful discussions.

This work was supported by Public Health Service grants AI21548 and AI28457 from the National Institute of Allergy and Infectious Diseases.

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