NOTES

Chromosomal System for Studying AmpC-Mediated β-Lactam Resistance Mutation in *Escherichia coli*

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In some enterobacterial pathogens, but not in *Escherichia coli*, loss-of-function mutations in the *ampD* gene are a common route to β-lactam antibiotic resistance. We constructed an assay system for studying mechanism(s) of enterobacterial *ampD* mutation using the well-developed genetics of *E. coli*. We integrated the *Enterobacter ampRC* genes into the *E. coli* chromosome. These cells acquire spontaneous recombination- and SOS response-independent β-lactam resistance mutations in *ampD*. This chromosomal system is useful for studying mutation mechanisms that promote antibiotic resistance.

Mutation is a primary cause of bacterial resistance to antibiotics. Some mutations promote resistance directly (e.g., quinolone resistance mutations in genes encoding its *Escherichia coli* targets, *gyrA* and *gyrB* [16]). Other chromosomal mutations can cause mutator phenotypes that increase the likelihood of acquiring a resistance mutation (42). Mutations also ameliorate the otherwise deleterious effects on cell growth and physiology of some antibiotic resistance-conferring mutations (22). Although antibiotic resistance has been studied intensively, the mechanisms that generate resistance mutations are poorly understood.

In addition to spontaneous mutation in exponentially growing cells (growth-dependent mutation), other mutation mechanisms exist that may pertain to antibiotic resistance (34, 36, 37). For example, factors such as antibiotic concentration (23), environmental conditions (12), or other stress-inducing phenomena (1, 34, 36) may enhance or repress mutational machinery that leads to resistance mutations (for a review, see reference 31). Some mutation mechanisms or factors may be more important when the organism is under suboptimal growth conditions, as is probably the case during certain stages of an infection. In this study, we utilize a relatively well-described β-lactam resistance pathway as a model system to begin dissecting the mechanism(s) of antibiotic resistance mutation using the tools of *E. coli* genetics.

Chromosomally encoded AmpC β-lactamases confer β-lactam antibiotic resistance in many pathogenic and opportunistic bacteria and are ubiquitous in enterobacteria, except for the salmonellae, klebsiellae, *Proteus mirabilis*, *Shigella flexneri*, and *Shigella dysenteriae* (30, 32). Their expression is inducible in all but *E. coli* and the shigellae (30). In inducible strains, *ampC* transcription is controlled by the transcriptional activator AmpR (2). AmpR activity is regulated allosterically by two cell wall components, 1,6-anhydromuropeptide and UDP-*N*-acetylmuramic acid-pentapeptide (UDP-MurNAc-pentapeptide). The first allows, and the second blocks, AmpR transcriptional activator activity at *ampC* (19). AmpD converts (activator-promoting) 1,6-anhydromuropeptide to (activator-blocking) UDP-MurNAc-pentapeptide, which then binds AmpR and blocks *ampC* transcription. Thus, loss-of-function mutations in *ampD* cause 1,6-anhydromuropeptide accumulation and constitutively induced AmpC β-lactamase production (7, 20, 21, 27). *ampD* missense and nonsense mutations are common in AmpC-mediated, β-lactam-resistant clinical isolates (25, 38). Also, some β-lactam antibiotics can induce expression of *ampC* by causing an increase in the cytoplasmic concentration of 1,6-anhydromuropeptide (7, 30).

E. coli lacks *ampR*, and low-level *ampC* expression results from a promoter embedded in the overlapping fumarate reductase (*frdABCD*) operon (13). High-level β-lactam resistance, mediated by *ampD* loss-of-function mutation, can be reconstituted in *E. coli* when the *ampR* and *ampC* genes of other enterobacteria are expressed from a plasmid (28, 35). We have integrated the *ampRC* genes from *Enterobacter cloacae* into the *E. coli* chromosome to assay *ampD* mutation, as selected by its β-lactam resistance phenotype. Background resistance imparted by the native *ampC* gene does not interfere with assays involving the reconstituted system. Integrating the *ampRC* genes into the chromosome improves upon previous plasmid-based *ampRC* expression systems by allowing genetic analyses not possible previously, first, because many mutant alleles used to study DNA repair, recombination, and mutation cause plasmid instability (e.g., reference 6). Second, the single-copy *ampRC* locus more closely models the situation in clini-

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TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Relevant genotype	Reference or source
Strains		
DM49	*lexA3*	29
FC526	Δ*recG263::kan*	11
GY8322	Δ(*srlR-recA*)*306*::Tn*10*	S. Sommer; ENZ280 (8) carrying the K5353 mini-F plasmid (9)
SMR821	*lexA3 malB*::Tn*9*	33
SMR1827	FC40 *sulA211*	33
SMR4562[a]	*rec⁺attλ⁺*	33; genotype identical to FC40 (4)
SMR4649	FC40 *sulA211 lexA51*	33
SMR5078	*recB21 recC22 sbcB15 sbcC201 hsdr_K⁻m_K⁺* (λ*xis1 c*Its857)	14
SMR5156	SMR4562 (λ*xis1 c*Its857)	SMR4562 × λSR446 (14)
SMR5201	*recB21 recC22 sbcB15 sbcC201 hsdrK⁻mK⁺* Δ*attλ::ampRC*	SMR5078 × DNA from pJP2
SMR5222	SMR4562 Δ*attλ::ampRC*	SMR 5156 × P1 SMR5201
SMR5225	SMR5222 Δ(*srlR-recA*)*306*::Tn*10*	SMR5222 × P1 GY8322
SMR5578	SMR5222 Δ*recG263::kan*	SMR5222 × P1 FC526
SMR5652	SMR5222 Δ*recG263::kan* Δ(*srlR-recA*)*306*::Tn*10*	SMR5578 × P1 GY8322
SMR5701	SMR1827 (λ*xis1 c*Its857)	SMR1827 × λSR446
SMR5702	SMR4649 (λ*xis1 c*Its857)	SMR4649 × λSR446
SMR5715	SMR5222 *lexA3 malB*::Tn*9*	SMR5222 × P1 SMR821
SMR5725	SMR5222 *sulA211 lexA51*	SMR5702 × P1 SMR5201
SMR5749	SMR5222 *sulA211*	SMR5701 × P1 SMR5201
Plasmids		
pEc1c	*E. cloacae ampRC⁺*	35
pJP2	pTGV-light *ampRC⁺*	This work
pJP19	pACYC184 *ampD⁺*	This work[b]

[a] Full genotype is Δ(*lac-pro*)XIII *thi ara* Rif^r [F′ α45, *lacI*^q *lacI33*Ω*lacZ*].

[b] Plasmid pJP19, carrying the *E. coli ampD⁺* gene and promoter, was created by amplifying *ampD⁺* from *E. coli* wild-type strain MG1655 (3) chromosomal DNA using primers AmpD no. 1, 5′-GGGTTTTCATGAGAGGCGGCATGTTAAAACTCCAG-3′; and AmpD no. 2, 5′-GGGTTTAAGCTTTCATGTTGTCTCCTTGCTGACCAG-3′. The primers incorporate terminal *Bsp*HI and *Hind*III restriction sites at the 5′ and 3′ ends (respectively) of the amplified fragment. Amplified *ampD⁺* DNA and pACYC184 DNA (5) were digested with *Bsp*HI and *Hind*III, and the *ampD⁺* fragment was ligated into pACYC184. pJP19-mediated *ampD* expression was confirmed by complementation to β-lactam sensitivity of four independent *ampD* β-lactam-resistant mutants.

cally relevant enterobacterial resistance (30). Third, this chromosomal system excludes mutations that confer β-lactam resistance by increasing plasmid (and, therefore, *ampC*) copy number.

An *ampRC* expression cassette in the *E. coli* chromosome was constructed as follows. The strains and plasmids used are shown in Table 1. SMR5222, an *E. coli* strain carrying the *E. cloacae ampRC⁺* genes in the *E. coli* chromosome, was constructed using the TGV (transgenic vector) system for integrating linear DNA cassettes into the *E. coli* chromosome (14). *E. cloacae* strain MHN1 *ampRC⁺* genes were isolated from plasmid pEc1c (35) by digestion with *Bam*HI and *Sal*I and were ligated into *Bgl*II- and *Xho*I-digested pTGV-light (14) plasmid DNA, creating pJP2. pJP2 was digested with *Nde*I to generate an *ampRC⁺* fragment flanked on both sides by homology to the *E. coli* attachment site for phage λ (*att*λ) for linear trans-

formation in the TGV system (14). SMR5201, a transformant carrying *ampRC⁺* replacing *att*λ (confirmed by PCR as described elsewhere [14]) was used as a P1 donor to move *att*λ::*ampRC⁺* into SMR5156 by P1 transduction (as described elsewhere [14]) to create SMR5222. Subsequent strains are isogenic to SMR5222 and were created using standard phage P1 transduction (referenced elsewhere [14]), and the constructions are outlined in Table 1.

We used the chromosome-based *ampRC* expression system to determine rates of spontaneous (growth-dependent) ampicillin resistance (Amp^r) mutation in otherwise isogenic strains

TABLE 2. Rates of β-lactam resistance mutation in *E. coli* DNA repair-deficient mutants

Relevant genotype[a] and expt[b]	Mutation rate (mutations/cell/generation, 10⁻⁷)	Rate relative to *rec⁺* within each expt	Mean mutation rate (mutations/cell/generation, 10⁻⁷) ± SE	Mean relative to *rec⁺* within each expt ± SE
rec⁺			1.4 ± 0.3	1.0
1	0.460	1		
2	2.18	1		
3	2.71	1		
4	1.10	1		
5	1.60	1		
6	0.632	1		
7	1.20	1		
recA			1.0 ± 0.3	0.66 ± 0.1
1	0.242	0.53		
2	0.908	0.42		
3	2.72	1.0		
4	0.693	0.63		
5	0.950	0.59		
6	0.277	0.44		
7	1.24	1.0		
recG			2.0 ± 0.8	1.0 ± 0.2
1	0.342	0.74		
2	3.20	1.5		
3	2.38	0.88		
recA recG			1.2 ± 0.5	0.65 ± 0.1
1	0.235	0.51		
2	1.70	0.78		
3	1.79	0.66		
lexA3			0.96 ± 0.2	0.79 ± 0.1
5	1.48	0.93		
6	0.362	0.57		
7	1.05	0.88		
sulA			1.0 ± 0.1	0.93 ± 0.1
5	1.05	0.66		
6	0.665	1.1		
7	1.29	1.1		
***sulA lexA5* (Def)**			1.7 ± 0.3	1.5 ± 0.04
5	2.45	1.5		
6	0.895	1.4		
7	1.69	1.4		

[a] Strains used were SMR5222, *rec⁺* (recombination and SOS response proficient); SMR5225, *recA* (recombination and SOS response deficient [40]); SMR5578, *recG* (recombination proficient, elevated for stationary-phase-mutation [11, 15]); SMR5652, *recG recA* (recombination, SOS, and stationary-phase mutation deficient [11, 15]); SMR5715, *lexA3* (recombination proficient, SOS gene induction defective [40]); SMR5749, *sulA* (allows viability in the presence of a *lexA*-null mutation [40]); and SMR5725, *sulA lexA51*(Def) (SOS-induced genes expressed constitutively [40]).

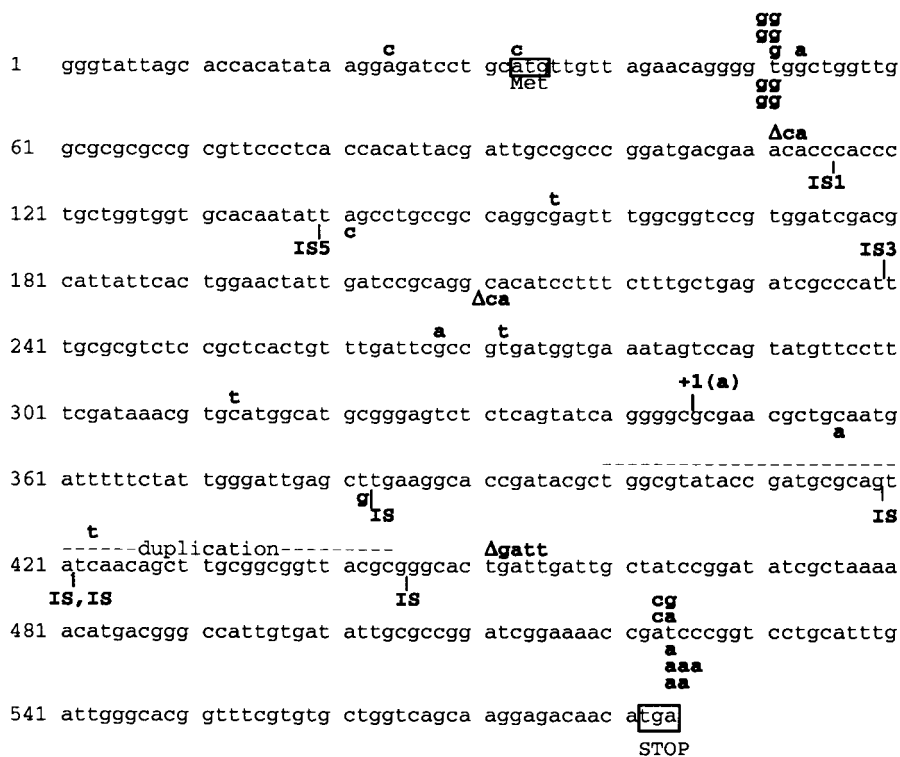[b] Data preceded by the same experiment number were gathered in parallel.

```
                                                                             gg
                                                                             gg
                                           c                 c                g  a
  1   gggtattagc accacatata aggagatcct gc|atg|ttgtt agaacagggg tggctggttg
                                            Met                               gg
                                                                              gg

                                                                     Δca
 61   gcgcgcgccg cgttccctca ccacattacg attgccgccc ggatgacgaa acacccaccc
                                                                      |
                                                                     IS1
                                              t
121   tgctggtggt gcacaatatt agcctgccgc caggcgagtt tggcggtccg tggatcgacg
                             |    c                                              |
                            IS5                                                IS3

181   cattattcac tggaactatt gatccgcagg cacatccttt ctttgctgag atcgcccatt
                                         Δca
                                   a        t
241   tgcgcgtctc cgctcactgt ttgattcgcc gtgatggtga aatagtccag tatgttcctt

                                                    +1(a)
               t
301   tcgataaacg tgcatggcat gcgggagtct ctcagtatca ggggcgcgaa cgctgcaatg
                                                                        a

361   atttttctat tgggattgag cttgaaggca ccgatacgct ggcgtatacc gatgcgcagt
                            g|IS                               --------------------------
                             IS                                                    IS
            t
      -----duplication--------         Δgatt
421   atcaacagct tgcggcggtt acgcgggcac tgattgattg ctatccggat atcgctaaaa
       |                          |
      IS,IS                      IS                           cg
                                                              ca
481   acatgacggg ccattgtgat attgcgccgg atcggaaaac cgatcccggt cctgcatttg
                                                              a
                                                              aaa
                                                              aa

541   attgggcacg gtttcgtgtg ctggtcagca aggagacaac a|tga|
                                                    STOP
```

FIG. 1. Location of *ampD* mutations. Forty independent Amp[r] mutants (20 from *rec*[+] strain SMR5222 and 20 from *recA* strain SMR5225) were screened for *ampD* mutations by complementation to β-lactam sensitivity using pJP19 (Table 1). Apparent *ampD* mutations were sequenced (Lone Star Labs, Houston, Tex.) from PCR products using primers AmpD no. 3, 5′-GCGCGTCTCCGCTCACTGTTT-3′; and AmpD no. 4, 5′-GCA TGCCATGCACGTTTATCG-3′. The PCR products were generated with primers AmpD no. 1 and AmpD no. 2 (legend to Table 1). The *E. coli* *ampD* sequence is given, along with 32 bp upstream of the ATG start codon. Mutations found in *rec*[+] mutants are indicated above, and mutations found in *recA* mutants are shown below, the sequence. IS, insertion sequence.

lacking various recombination and SOS genes. Growth-dependent mutation rates were measured in 15 tube fluctuation tests. Fifteen independent cultures for each strain were grown to saturation in 5 or 10 ml of Mueller-Hinton (MH) broth (Difco), shaking at 37°C. Cultures were diluted 10-fold, and 50 μl was plated in duplicate on MH agar plates containing 100 μg/ml ampicillin. The plates were incubated overnight at 37°C, and Amp[r] colonies were counted. Viable cell counts of the saturated cultures were from dilutions plated on MH incubated overnight at 37°C. Mutation rates were calculated by the method of the median (26).

The recombination and SOS genes examined—*recA*, *recG*, *recA recG*, *lexA3*(Ind[−]), *sulA*, and *sulA lexA*(Def)—are not required for most growth-dependent mutation; however, they are required for a mechanism of mutation observed under growth-limiting conditions of carbon starvation (for a review, see reference 37; see also references 41 and 43). Moreover, the SOS response controls several mutation-promoting proteins (40) whose possible involvement in β-lactam resistance mutation we wished to test. The β-lactam resistance mutation rate in recombination- and SOS-proficient (*rec*^{+}) cells is about 1.4 × 10^{−7} cell[−1] generation[−1] (Table 2). The strains tested displayed only small differences in growth-dependent mutation rates, indicating that recombination and SOS genes are not important for most growth-dependent β-lactam resistance mutation (Table 2).

The following experiments demonstrated that the β-lactam resistance mutations are in *ampD*. Based on prior observations in an *E. coli* model and in clinical isolates of enterobacterial pathogens (7, 27), we expected most of the β-lactam resistance mutations to be in *ampD*. To test this, 20 independent ampicillin resistant mutants (each from a separate independent culture) from the *rec*[+] and *recA* fluctuation test experiments were examined further. β-Lactam sensitivity was restored to each of the 40 mutants by transforming each with pJP19 (Table 1) carrying the *ampD*[+] gene. This complementation test shows that the ampicillin resistance mutations of the 40 independent mutants are recessive, loss-of-function mutations in *ampD*.

We determined the *ampD* sequences for each of the 40 mutants. Little difference was found between the *rec*[+] and *recA* backgrounds (Fig. 1), suggesting similar mutation mechanism(s) in each. Many different mutations and insertions in *ampD* conferred AmpC-mediated β-lactam resistance.

Among the eight substitution mutations identified here, two were identified previously from β-lactam resistant isolates. A Trp7Gly substitution in AmpD occurred in both the *rec*[+] and *recA* strains (Fig. 2) and previously in *E. coli* ampicillin-resistant mutants of cells expressing the *E. cloacae ampRC* genes from a plasmid (18). Also, we found Asp164Glu and Asp164Ala in both *rec*[+] and *recA*. Asp164Glu was found previously in *Citrobacter freundii* (39). Both previous *ampD* mutations were shown to cause full derepression of *ampC*, as *ampD*-null mutations do (28, 39).

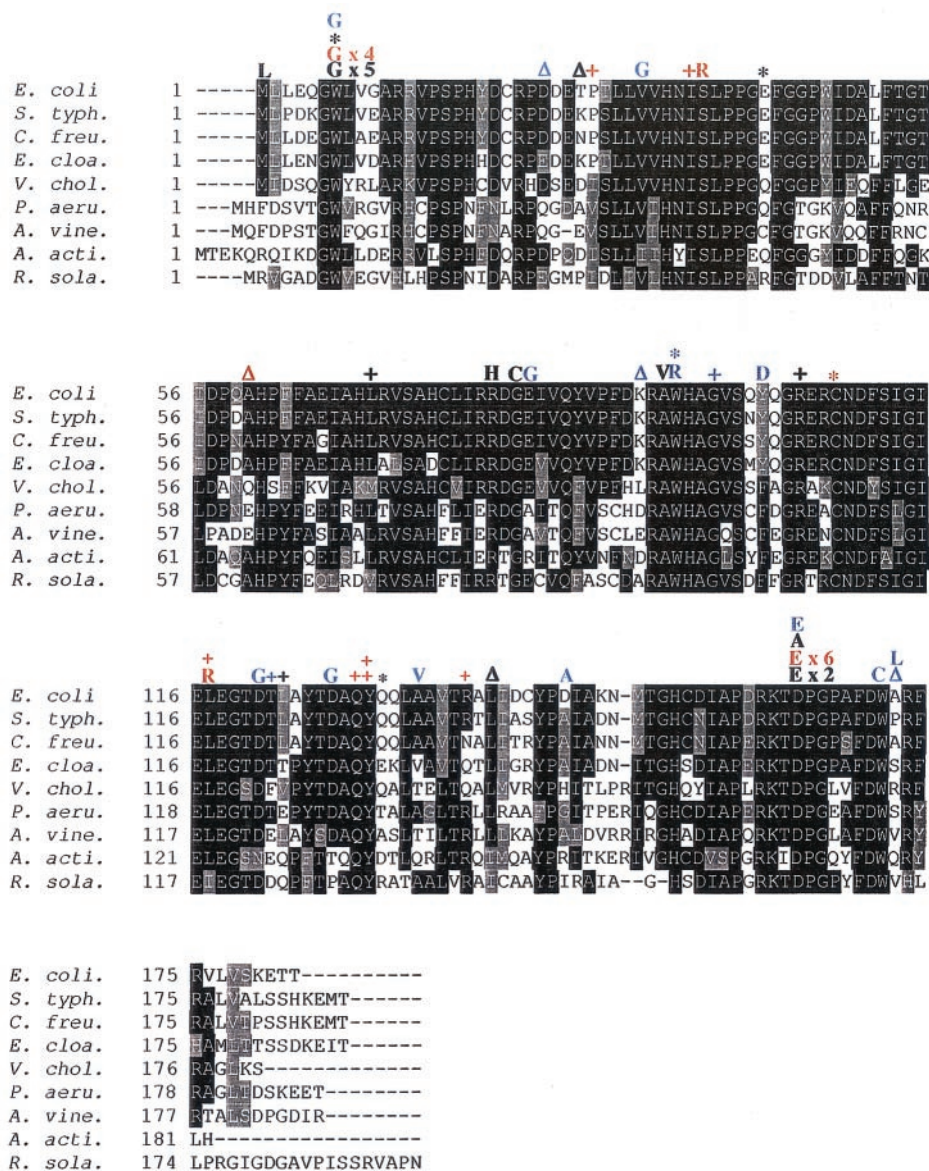Other *ampD* substitution mutations found include

FIG. 2. Amino acid substitutions, insertions, and deletions identified in the *rec*[+] and *recA* mutants aligned with the *ampD* genes from nine enterobacteria. Also included are previously identified *ampD* mutant proteins from other laboratories (10, 18, 24, 28). *rec*[+] mutations are in black, *recA* mutations are in red, and previously identified mutations are in blue. Insertions are indicated by a plus sign (+), deletions are indicated by a delta (Δ), and nonsense mutations are indicated by an asterisk (∗). Mutations isolated multiple times show the number of times that each was encountered for each strain (*rec*[+] or *recA*). Abbreviations: *S. typh*, *Salmonella enterica* serovar Typhimurium; *C. freu*, *C. freundii*; *E. cloa*, *E. cloacae*; *V. chol*, *Vibrio cholerae*; *P. aeru*, *Pseudomonas aeruginosa*; *A. vine*, *Azotobacter vinelandii*; *A. acti*, *Actinobacillus actinomycetemcomitans*; and *R. sola*, *Ralstonia solanacearum*. Alignment was performed using ClustalW (17) and formatted using BOXSHADE.

Ser37Arg, Arg80His, Gly82Cys, Ala94Val, and Leu117Arg (Fig. 2). Although these might or might not inactivate *ampD* fully, substitutions that alter or abolish AmpD function reveal amino acids that are important for AmpD structure and/or function. Conservative substitutions, such as Ala94Val or Asp164Glu, highlight the specific steric and/or chemical requirements of the wild-type amino acids. For example, the intolerance for Ala94Val suggests that the smaller size of alanine is important here, because both amino acids are similarly hydrophobic. The Asp164Glu substitution involves similar charges, suggesting that this amino acid position makes important catalytic or structural contacts disrupted by the larger

glutamic acid side chain. Asp164 is probably not simply a surface amino acid, because it has a seemingly stringent size requirement and because alanine at this position is also not tolerated.

Alignments show that *ampD* is highly conserved among various bacteria (e.g., Fig. 2). Asp164, Ala94, and the other substituted amino acids from our mutation studies are highly conserved among the aligned *ampD* sequences (Fig. 2) and further highlight their potential structural and/or functional importance.

The variety of loss-of-function mutations observed in this system suggests its utility for studying many kinds of mutation

mechanisms. This system may be useful additionally for studying the forward mutation spectra caused by potential damaging agents and environmental factors, because mutations in the small *ampD* gene are easily selected and sequenced.

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