Accumulation of large non-circular forms of the chromosome in recombination-defective mutants of Escherichia coli

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Abstract

Background: Double-strand breakage of chromosomal DNA is obviously a serious threat to cells because various activities of the chromosome depend on its integrity. However, recent experiments suggest that such breakage may occur frequently during "normal" growth in various organisms – from bacteria through vertebrates, possibly through arrest of a replication fork at some endogenous DNA damage.

Results: In order to learn how the recombination processes contribute to generation and processing of the breakage, large (> 2000 kb) linear forms of Escherichia coli chromosome were detected by pulsed-field gel electrophoresis in various recombination-defective mutants. The mutants were analyzed in a rich medium, in which the wild-type strain showed fewer of these huge broken chromosomes than in a synthetic medium, and the following results were obtained: (i) Several recB and recC null mutants (in an otherwise rec+ background) accumulated these huge linear forms, but several non-null recBCD mutants (recD, recC1001, recC1002, recC1003, recC1004, recC145, recB154, and recB155) did not. (ii) In a recBC sbcA background, in which RecE-mediated recombination is active, recA, recF, recQ, recE, recT, recO, and recR mutations led to their accumulation. The recJ mutant accumulated many linear forms, but this effect was suppressed by a recQ mutation. (iii) The recA, recJ, recQ, recF and recR mutations led to their accumulation in a recBC sbcBC background. The recJ mutation showed the largest amount of these forms. (iv) No accumulation was detected in mutants affecting resolution of Holliday intermediates, recG, ruvAB and ruvC, in any of these backgrounds.

Conclusion: These results are discussed in terms of stepwise processing of chromosomal double-strand breaks.

Background

Double-strand (ds) breakage of chromosomal DNA is obviously a serious threat to cells because various activities of the chromosome – gene expression, replication and partition – depend on its integrity. However, recent experiments suggest that such chromosomal ds breakage may occur relatively frequently during "normal" growth in several organisms – in bacteria [1,2], yeast [3] and chicken cells [4].

In Escherichia coli, spontaneous breakage and degradation of the chromosome associated with a replication fork were predicted from early genetic analysis and were detected under various conditions of altered replication (for
defective mutants in the chromosome obtained from various recombination-phoresis procedure to measure large non-circular forms of In this work, we employed the pulsed-field gel electrophoresis. In our analysis, growing E. coli cells are harvested, embedded in an agarose plug, and lysed in situ. The chromosomes in a plug are electrophoresed in varying electric fields. An example of such a gel is shown in Figure 1. The DNA is partitioned in three places in the gel – the well, the area just below the well (marked by a bar to the right of the gel), and the lower area. Intact circular chromosomes stay in the well likely because they are trapped in the branches of agarose resin. Large linear forms generated by a ds break in this circle would escape from agarose trap and form broad bands beneath the well (marked by the bar). This area corresponds to unbranched linear forms DNA of more than 2000 kb when compared with yeast chromosome markers. When the DNAs become smaller by degradation, they will migrate further. These interpretations are based on a previous work with a yeast circular chromosome and our analysis of E. coli chromosomal breakage after loss of restriction-modification genes. In this work, we focus on the second DNA species – the huge linear forms in the area just below the well (Figure 1, marked by the bar).

In the experiment shown in Figure 1, a rec+ strain (in AB1157 background) grown in minimal medium (M9) (Figure 1, lane 2) gave rise to some of these huge linear DNAs in this area. There was less of this DNA species when the cells were grown in a rich medium (LB) (Figure 1, lanes 3). In an isogenic recB21 recC22 strain, the amount was larger than in rec+

We do not know why the medium makes such a difference. It could reflect properties of the spontaneous DNA damages, the replication fork, the number of replication forks, the number of chromosomes, the organization and structure of the chromosomes, the repair machinery, or the availability of homologous chromosomes for repair. All of these features will influence the chromosome stability not only in rec+, but also in mutants. This medium-dependence is in the opposite direction to what is simply expected from generation of a double-stranded chromosomal end by collapse of a replication fork with another, replication fork moving in the same direction, because replication initiation should be more frequent in a rich medium than in a poor medium. Whatever the reason, we chose to use the rich medium in which the rec+ strain produce less linear forms, because the background is clear and may allow sensitive detection of their increase in a survey of various recombination-defective mutants.
The accumulation of huge linear DNAs was also seen in other recBC null mutants in this AB1157 genetic background (recB21, recC22 and recC73 (Figure 2A)) and in another, V66, genetic background (recC73 (Figure 2B)), as observed earlier [1,2]. A recD mutant showed no accumulation. The other non-null recBCD alleles examined (recC1001, recC1002, recC1003, recC1004, recC2145, recB2154, and recB2155) did not accumulate the huge linear forms (Figure 2B). We do not know why the same mutant allele, recC73, shows more accumulation in V66 background than in AB1157 background in a reproducible manner (Figures 2A and 2B).

The other mutants tested – recA, recE, recG, recJ, recN, recO, recQ, recR, ruvAB, and ruvC – did not accumulate huge linear DNAs. The recF mutation partially suppressed the effect of the recC73 mutation in accumulating the huge linear chromosomes (Figure 2B).

recBC sbcA background

In the recBC sbcA strain, an sbcA mutation on the Rac prophage expresses recET genes, which promotes homologous recombination at a ds end [7]. The accumulation of the huge linear was seen with recA, recE, recT, recf, recQ, recF, recO and recR strains (Figure 2C). Mutations in genes involved in processing Holliday structures – recG, ruvAB and ruvC – did not lead to their accumulation. The accumulation by recI mutation was suppressed by a recQ mutation (Figure 2C, lanes 7 and 15).

recBC sbcBC background

In the recBC sbcBC strain, RecBCD enzyme is inactive and RecFOR and RecQJ proteins promote recombination together with RecA [19]. In the recBC sbcBC background, recA, recF, recJ, recQ and recR mutants accumulated these huge linear to varying extents (Figure 2D). However, the ruvC mutation did not lead to accumulation.

Control experiments

These assays were carried out more than twice for each strain, and the extent of accumulation of the linear forms was reproducible. The DNA in the area just below the origin was also measured by densitometry to confirm the above results (data not shown).

When the chromosomal DNA in the agar plug was digested with a restriction enzyme (XbaI) before the pulsed-field gel electrophoresis, all the strains examined produced comparable amounts of DNA (Figure 3). This amount is much larger than the large linear forms. This indicates that the total amount of undegraded DNA associated with the cells is comparable for all the strains.

Discussion

We found that large, non-circular forms of the chromosome accumulate in varying amounts in various recombination-defective mutants of Escherichia coli. Our operational definition of the non-circular forms is their presence in an area just below the well in our pulsed-field gel, as marked by a bar in Figure 1. The molecular species in this area may not be limited to a simple linear form of varying lengths. If a chromosome carries multiple replication forks as usual at 37°C in rich media, more than one double-strand break may be necessary to form a non-circular, branched species, which should be able to move through the gel. Finding out macroscopic forms of these giant molecules would be a technical challenge (see [20], for example). We do not know why DNAs make two broad bands in this area (Figure 1, 4th lane, for example), either. Depending on the electrophoresis condition, one narrow band, a pair of two bands or one very broad band was observed (data not shown).
Figure 2
Accumulation of large non-circular forms of the chromosome in recombination-defective mutants. A: In an otherwise rec+ background. The mutation alleles are as follows: ΔrecA306::Tn10, recB21 recC22, recB268::Tn10, recC266::Tn10, recC73, recD1901::Tn10, recF143, recG258::mini-Tn10 Kan, recJ284::Tn10, recN1502::Tn5, recQ1801::Tn3, recQ1803::Tn3, recR252::mini-Tn10 Kan, ΔnuvAB100::Cm, ΔnuvC100::Cm, nuc53 eda::Tn10. B: Various recBCD alleles in V66 background. C: In a recBC sbcA background. The mutation alleles are the same as in A except for recE159, recQ1801, recT101::Tn10, and ΔnuvAB::Tc. D: In a recBC sbcBC background. The mutation alleles are the same as in A except for recN262 tyrA16::Tn10.
Abundance of these huge non-circular forms is expected to be affected by several factors, which might work potentially in opposite directions, such as: (i) breakage in the cell; (ii) degradation in the cell; (iii) repair in the cell; (iv) breakage and degradation out of the cell. Each term is, in turn, affected by other factors such as chromosome organization, number of the replication forks, speed of the replication forks, abundance of specific proteins, and so forth. Therefore, our finding of accumulation of more of the non-linear forms in a rich medium than in a poor medium (Figure 1) does not immediately allow us to conclude that starving conditions induce a chromosomal double-strand breakage.

Spontaneous DNA damages, repair and degradation are expected to be the key processes in interpreting our data. Spontaneous DNA damages may interfere with replication fork progression and produce chromosomal double-stranded breaks. This would lead to extensive exonucleolytic degradation. Complete repair at some of these steps would reconstitute a circular chromosome, which will stay in the well. On the other hand, further degradation of the huge, non-circular forms would result in shorter or no fragments, which will run faster in the gel. The presence of huge linear forms, therefore, probably indicates both the absence of complete repair and the absence of further degradation. The absence of the large linear forms could either mean the presence of complete repair or the presence of extensive degradation activity. Our control experiments demonstrated that restriction digestion of chromosome DNAs before the electrophoresis results in release of comparable amounts of DNA from the wells in all the strains examined (Figure 3). This result, at least, excludes the possibility that the absence of the large, non-circular chromosomes in some strains (Figure 2) reflects the absence of DNAs in the wells during the process or by extensive and general nuclease action. Of course, we cannot exclude the possibility that the broken chromosomes specifically have suffered extensive degradation.

In spite of these potential complexity and essential ambiguity, our measurements provided a unique clue to the action of recombination-associated enzymes in the chromosome metabolism. Indeed, some of our observations in the mutants can be readily related to the established properties of the affected enzyme.

Accumulation of the huge linear DNAs in the recBC null mutants can be interpreted from the known properties of RecBCD enzyme in a straightforward way. These null mutant enzymes cannot degrade DNA from a ds break nor can they repair DNA by recombination [6]. We assume that they cannot repair the broken chromosomes to form intact circular chromosomes and that they cannot degrade them into smaller pieces. The recD mutant does not show nuclease activity but is recombination-proficient and able to repair the broken DNA molecules [6]. This explains why it does not accumulate the huge linear forms. The other non-null recBCD mutants (recC1001, recC1002, recC1003, recC1004, recC2145, recB2154, and recB2155) are all nuclease positive [21,22]. They would be expected to degrade the huge linears. They retain some to nearly complete recombination proficiency [21,22], which may contribute to repair of the large linears into circles. The

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**Figure 3**

**Pulsed-field gel electrophoresis of the chromosomes after restriction enzyme digestion.** The cells were lysed in an agarose plug and were treated with Xbal before pulsed-field gel electrophoresis. M indicates yeast chromosome marker.
other recombination-defective mutants, in otherwise rec− background, did not accumulate the huge linears probably because the DNA was degraded by active RecBCD enzyme or was not produced.

Partial suppression of the accumulation of the huge linears in a recBC null allele by a recF mutation (Figure 2B) leads to several possible explanations. For example, RecF-mediated homologous recombination may transform a circular chromosome, possibly with a spontaneous damage, into some type of non-circular forms. This is expected because RecF-mediated recombination is non-conservative in the sense that it generates only one progeny DNA molecule from two parental DNA molecules [10]. Alternatively, RecF function may somehow help generation of broken chromosomes or maintenance of break to load RecA [23].

In the recBC sbcA and the recBC sbcBC backgrounds, the absence of RecBCD nuclease may prevent faster degradation of the large non-circular DNAs. However, we see only little accumulation of the broken forms. One might expect that the accumulation of the huge linears may correlate with the capacity for recombination repair that reconstitutes a circular form. Indeed, the effects of recA, recJ and several other rec mutations on accumulation of the huge linear chromosomes in these two recBC backgrounds (Figures 2C and 2D) were similar to their negative effects on conjugal recombination [19] with interesting exceptions (see next paragraph). This accords with the concept that a huge linear fragments of the chromosome is involved in recombination following conjugation. However, any of the recombination mutants that lead to accumulation of linear DNA could affect the probability of breaks occurring in the first place.

The mutations in Holliday-structure-processing enzymes – RecG, RuvAB, and RuvC – did not result in accumulation of the huge linears even in the recBC-minus background. The complex intermediate forms accumulating in these mutants may be trapped in the agarose gel (see [24,25]). An alternative interpretation could be that these enzymes may be involved in generation of double-strand breaks as hypothesized by Seigneur et al. [17].

The accumulation by the recJ mutation in the recBC sbcA background is suppressed by a recQ mutation (Figure 2C). Kusano et al. [26] found that both sensitivity to DNA damaging agents and decreased association of crossing-over with double-strand break repair in a recBC sbcA recJ strain are suppressed by mutant recQ alleles. Such suppressing relationship was interpreted to suggest that RecQ acts prior to or concurrently with RecJ. Pulsed-field gel electrophoresis analysis of chromosomes after ultraviolet irradiation has revealed extensive chromosome degrada-

tion dependent on uvrA incision enzyme [27]. A report [28] showed that RecQ and RecI proteins process nascent DNA at replication forks blocked by ultraviolet irradiation prior to the resumption of DNA synthesis (see also [29]).

The accumulation of the non-circular, broken chromosomes correlated with the growth rate or DNA damage response in most of the recBC-minus background [30]. The recB or recC null mutation showed low viability even in the absence of exogenous DNA damage [31,32]. A simple interpretation of these data is that RecA, RecFOR, and RecQJ functions (and RecET functions for the sbcA background) repair chromosome breakage and/or prevent generation of the breakage. The major contradiction observed here is the phenotype in ruv mutants. The ruv mutants in all the background did not show any accumulation of the broken chromosome. This may suggest that the possible role of Ruv protein is making a break into dsDNA [33].

Conclusions

Our sensitive measurements of the large non-circular forms of the chromosome – which should be able to detect one ds break out of 4 million bp – provided unique sets of data that would help in further elucidating the mechanisms of chromosome double-strand break repair. A simplest interpretation of our data is that RecBCD enzyme is involved in repair and degradation of broken chromosomes, and that RecA, RecFOR, RecQJ and RecET functions are involved in prevention and/or repair of the breakage. Interaction was observed between a recC mutation and a recF mutation and between a recQ mutation and a recJ mutation. ruvABC mutants and a recG mutant did not accumulate broken chromosomes. Further molecular analysis would bring about interpretation of the present data in detailed molecular terms.

Methods

Bacteria

Escherichia coli K-12 strains used are listed in Table 1.

Media

E. coli cells were grown in M9 medium (1 × M9 salts [34], 0.2% glucose, 0.05 mM CaCl2, 0.5 mM MgSO4, 0.2% casamino acids and 1 microgram/ml vitamin B1) and LB broth (1.0% Bacto-tryptone, 0.5% Yeast extract and 1.0% NaCl) with antibiotics at the following concentrations when necessary: ampicillin (Amp) at 50 microgram/ml together with methicillin at 200 microgram/ml, chloramphenicol (Cml) at 25 microgram/ml, kanamycin (Kan) at 10 microgram/ml and tetracycline (Tet) at 10 microgram/ml.
### Table 1: Bacterial strains used here

| Strain   | Other name | Genotype                                                                 | Source/Reference |
|----------|------------|---------------------------------------------------------------------------|------------------|
| AB1157   | BIK788     | thr-1 leu-6 thi-1 lac<sup>+</sup> galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE<sup>3</sup> str-33 tax-33 supE<sup>44</sup> rec<sup>+</sup> | [36]             |
| TES1     | BIK733     | As AB1157, but recB268::Tn10                                               | K. Yamamoto [37] |
| JC5519   | BIK751     | recB268::Tn10                                                              | T. Kato [38]     |
| NZ101    | BIK2876    | recB268::Tn10                                                              | R. Lloyd [39]    |
| NZ103    | BIK2877    | recB268::Tn10                                                              | R. Lloyd [39]    |
| BIK3961  | BIK786     | As AB1157, but recB268::Tn10                                               | P1 (BIK2876) to AB1157 |
| BIK3963  | BIK787     | As AB1157, but recB268::Tn10                                               | P1 (BIK2877) to AB1157 |
| BIK806   | BIK787     | As AB1157, but recB268::Tn10                                               | [40]             |
| JC9239   | BIK783     | As AB1157, but recB268::Tn10                                               | A. J. Clark      |
| BIK1538  | BIK787     | recB268::Tn10                                                              | P1 (BIK400) to AB1157 |
| JC1213   | BIK787     | recB268::Tn10                                                              | A. Clark [41]    |
| BIK2563  | BIK787     | recB268::Tn10                                                              | P1 (BIK787) to AB1157 |
| BIK2565  | BIK787     | As AB1157, but recB268::Tn10                                               | P1 (BIK1044) to AB1157 |
| KEN24    | BIK1179    | recB268::Tn10                                                              | K. Yamamoto [40] |
| KD2216   | BIK1048    | recB268::Tn10                                                              | H. Nakayama [42] |
| BIK2680  | BIK788     | As AB1157, but recB268::Tn10                                               | P1 (BIK1048) to AB1157 |
| BIK2577  | BIK787     | As AB1157, but recB268::Tn10                                               | P1 (BIK1399) to AB1157 |
| HRS1004  | BIK1331    | .ruvAB::Tc                                                                 | T. Shiba & H. Shinagawa |
| HRS2302  | BIK1620    | As AB1157, but .ruvAB100::Cm                                                | H. Shinagawa [24]|
| HRS1100  | BIK1618    | As AB1157, but .ruvC100::Cm                                                 | H. Shinagawa [43]|
| KEN72    | BIK1051    | As AB1157, but ruvC33 eda::Tn10                                             | K. Yamamoto      |
| JC8679   | BIK813     | As AB1157, but recB268::Tn10                                               | A. J. Clark [44] |
| BIK1415  | BIK784     | As JC8679, but .recA306::Tn10                                              | [26]             |
| JCB619   | BIK784     | As JC8679, but recB143                                                     | A. J. Clark [44] |
| JCB9610  | BIK786     | As JC8679, but recB143                                                     | A. J. Clark [44] |
| N2796    | BIK1400    | As JC8679, but recB268::Tn10                                               | R. Lloyd [45]    |
| BIK814   | BIK784     | As JC8679, but recB268::Tn10                                               | Kusano et al. (1994b) |
| BIK1044  | BIK786     | As JC8679, but recB1502::Tn5                                               | Takahashi et al. (1993) |
| BIK1192  | BIK789     | As JC8679, but recG::Tn5                                                    | [26]             |
| RDK1693  | BIK1401    | As JC8679, but recB143                                                     | S. Lovett [46]   |
| BIK1427  | BIK784     | As JC8679, but recB143                                                     | [26]             |
| BIK1224  | BIK784     | As JC8679, but recB143                                                     | [26]             |
| AM265    | BIK1399    | As JC8679, but recB268::mini-Tn10 KAN                                       | R. Lloyd [47]    |
| BIK3884  | BIK784     | As JC8679, but recT101::Tn10                                               | N. Kobayashi-Takahashi |
| BIK1478  | BIK787     | As JC8679, but .ruvAB::Tc                                                   | P1 (BIK1331) to JC8679 |
| BIK1050  | BIK784     | As JC8679, but ruvC33 eda::Tn10                                             | [26]             |
| JCS623   | BIK752     | As AB1157, but recB268::Tn10                                               | T. Kato [48,49]  |
| BIK2176  | BIK762     | As JC7623, but .recA306::Tn10                                              | P1 (BIK733) to JC7623 |
| JCB111   | BIK762     | As JC7623, but recB143                                                     | A. J. Clark      |
| BIK1772  | BIK762     | As JC7623, but recB268::Tn10                                               | P1 (BIK814) to JC7623 |
| BIK1212  | BIK762     | As JC7623, but recB268::Tn10                                               | [10]             |
| JSB1774  | BIK762     | As JC7623, but recB268::Tn10                                               | P1 (BIK1224) to JC7623 |
| BIK1776  | BIK762     | As JC7623, but recB268::Tn10                                               | P1 (BIK1399) to JC7623 |
| KEN87    | BIK1181    | As JC7623, but ruvC33 eda::Tn10                                             | K. Yamamoto      |
| V66      | BIK796     | recB268::Tn10                                                              | A. Taylor [21]   |
| V68      | BIK796     | As V66, but recB268::Tn10                                                  | G. Smith [50]    |
| V73      | BIK1275    | As V66, but recB268::Tn10                                                  | G. Smith [21,50] |
| V69      | BIK1275    | As V66, but recB268::Tn10                                                  | G. Smith [21,50] |
| V69      | BIK1272    | As V66, but recB268::Tn10                                                  | G. Smith [21,50] |
| V71      | BIK1273    | As V66, but recB268::Tn10                                                  | G. Smith [21,50] |
| V72      | BIK1274    | As V66, but recB268::Tn10                                                  | G. Smith [21,50] |
| V1296    | BIK1910    | As V66, but recB268::Tn10                                                  | G. Smith [22]    |
| V1360    | BIK1911    | As V66, but recB268::Tn10                                                  | G. Smith [22]    |
| V1363    | BIK1912    | As V66, but recB268::Tn10                                                  | G. Smith [22]    |
| BIK1288  | BIK1288    | As BIK1288 (tet<sup>+</sup>)                                               | [51]             |
| BIK3713  | BIK1288    | As BIK1288 (tet<sup>+</sup>)                                               | tet<sup>+</sup> selection from BIK1288 |
| BIK3713  | BIK1288    | As BIK1288 (tet<sup>+</sup>)                                               | N. Kleckner via A. Taylor |
| BIK3732  | BIK2411    | As BIK2411, but argB1::Tn10                                                | P1 (BIK800) to BIK2411 |
| BIK3738  | BIK3738    | As BIK3738, but recB268::Tn10                                               | P1 (BIK3732) to BIK3713 |
Table 1: Bacterial strains used here (Continued)

| BIK4034 | BIK1276 | BIK1286 | BIK1290 | BIK1282 | BIK1284 | BIK2445 | BIK2446 | BIK2447 |
|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| A211 | As AB1157, but recC73 argA81::Tn10 | IN (rrnD-rrnE) l: F' lacZ20 yamB* recF* zic::Tn10 | As BIK1275, but recF* zic::Tn10 | As BIK1272, but recF* zic::Tn10 | As BIK1273, but recF* zic::Tn10 | As BIK1274, but recF* zic::Tn10 | As BIK1910, but recF* zic::Tn10 | As BIK1911, but recF* zic::Tn10 | As BIK1912, but recF* zic::Tn10 |

Preparation of DNA samples in agarose gel

The sample plugs were placed in the wells of a running gel (1.0% (w/v) SeaKem GTG agarose (FMC)) and solidified with molten 1.0% agarose. Pulsed-field gel electrophoresis was carried out in a Pharmacia/LKB apparatus under the following conditions: electrophoresis buffer, 1 x TBE (45 mM Tris-borate/1.25 mM EDTA); 165V; pulse time, 120 sec; run time, 24 hrs; temperature, 10°C. As a size marker, a plug containing yeast (Saccharomyces cerevisiae) chromosomes (Pharmacia) was used. After the run, the gel was stained with ethidium bromide and photographed under ultraviolet illumination. The DNA in the region of the huge linear chromosomes was quantified using a VILBER LOURMAT apparatus with BIO-PROFIL software.

The control experiment (XbaI digestion before the run) was done in a CHEF-DR III system (Bio-Rad) under the following conditions: electrophoresis buffer, 0.5 x TBE; 6 V/cm; angle, 120°; pulse time, 4 x 50 sec; run time, 20 hrs; temperature, 14°C. After the run, agarose gels were processed as described above.

Authors’ contributions

NH carried out all of the experiment and drafted the manuscript. IK supervised the work and edited the manuscript. All authors read and approved the final manuscript.

List of abbreviations

ds, double-strand. E. coli, Escherichia coli. Amp, ampicillin. Cml, chloramphenicol. Kan, kanamycin. Tet, tetracycline.

Acknowledgements

We are grateful to those listed in Table 1 for generous gift of materials, to Kohji Kusano and John Clark for comments on manuscript, and to Steve Kowalczykowski for discussion. The work was supported by grants (Tenkai, Repair, Genome Science, Genome Biology, Kiban, Genome Osmosis, National Project on Protein Structural and Functional Analyses) from MEXT of the Japanese government and by a grant from Uehara Memorial Foundation. NH was supported by JSPS Research Fellowship for Young Scientists and JSPS Postdoctoral Fellowships for Research Abroad.

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