EVIDENCE THAT TWO MAJOR REPLICONS COMPRISE THE GENOME OF STAPHYLOCOCCUS AUREUS

ROBERT A. ALTENBERN
Biological Sciences Laboratory, Fort Detrick, Frederick, Maryland

Received October 16, 1967

The genome of Escherichia coli and probably other enterics exists as a single but circular DNA molecule constituting a single replicon (Jacob and Wollman, 1961). The chromosome of Bacillus subtilis also exists as a single DNA molecule and behaves as a continuous linkage group within the confines of the replication origin and terminus (Yoshikawa and Sueoka, 1963). Investigations in this laboratory have led to a method for mapping the chromosome of Staphylococcus aureus by synchronizing chromosomal replication. Gene frequency analysis methodology developed by Sueoka and associates has been applied to Staph. aureus and supports the genomic map obtained by the synchronous chromosomal replication method. However, certain anomalies arose during derivation of a chromosomal map by gene frequency analysis which were inconsistent with the concept of a single replicon for the total genome of Staph. aureus. This report presents evidence that the genome of this organism is composed of two major replicons, one of which is present in a significantly greater number of copies than the other during early log phase.

MATERIALS AND METHODS

The strain of Staph. aureus employed in this study was isolated from clinical material and was maintained on Trypticase soy (TS) agar (Baltimore Biological Laboratories) slants. Singly auxotrophic mutants of this strain were isolated following exposure of the wild type to nitrosoguanidine. All broth media were Trypticase soy (TS) broth. Minimal agar was hydrolyzed casein medium whose composition was previously described (Altenbern, 1966).

Broth cultures (5 ml/tube) were inoculated with 0.1 ml of a 1:10 dilution of an overnight culture in TS broth and incubated on a shaker at 37 C. After 2, 3, 4, and 5 hr of incubation the optical densities at 650 mμ were 0.06, 0.25, 0.48, and 0.75 respectively for the wild type.

The synchronous chromosomal replication method of genomic mapping has been published (Altenbern, 1966) and has subsequently been modified to allow 120 min for chromosomal duplication (to be published). Gene frequency analysis methodology for Staph. aureus gene order is similar to the method of Yoshikawa and Sueoka (1963).

The relative gene frequencies of antibiotic resistance induced by nitrosoguanidine were determined for late log cells after exposure to 0.40% phenylethyl alcohol for 2 hr. Synthesis of DNA in these cells has been halted by phenyethyl alcohol, presumably, after completion of chromosomal duplication (Treick and Konetzka, 1964; Altenbern, 1966). The relative gene frequencies of antibiotic resistance induced by nitrosoguanidine in 2, 3, 4, and 5 hr cultures were normalized to the values obtained for the phenylethyl alcohol treated "reference cells". The normalized values of pairs of antibiotic resistance markers were used to construct the gene order as presented in Table 1.

Plating medium for detection of inhibitor-resistant mutants was TS agar containing any one of the following inhibitors: chloramphenicol, 4 μg/ml; novobiocin, 2 μg/ml; nitrofurazone, 8 μg/ml; acriflavin, 25 μg/ml. All platings were performed in triplicate or quadruplicate.

RESULTS

The locations of the genes to be considered here are presented in Fig. 1, as determined by the synchronous chromosomal replication technique wherein chromosomal duplication requires 120 min. The acriflavin resistance locus duplicates at the same time as the novobiocin resistance locus. Gene frequency experiments indicated that more than one "growing point" occurred during chromosomal replication in early log phase (Table 1). The gene controlling acriflavin resistance apparently shifted position when the gene order of 2 hr cells

TABLE 1. Gene order on Staph. aureus chromosome obtained by gene frequency analysis on cells of different ages.

	Age of cells			
	2 hr	4 hr	5 hr	6 hr
ACR/NOV	5.8	1.3	0.68	0.42
ACR/NFN	6.37	1.36	2.0	2.54
CMP/NOV	3.0	1.94	1.05	1.39

All values given are normalized to the relative gene frequencies of the phenylethyl alcohol treated reference cells.

Gene order at 2 hr. ACR-CMP-NOV NFN

Gene order at 5 hr. CMP-NOV-ACR-NFN

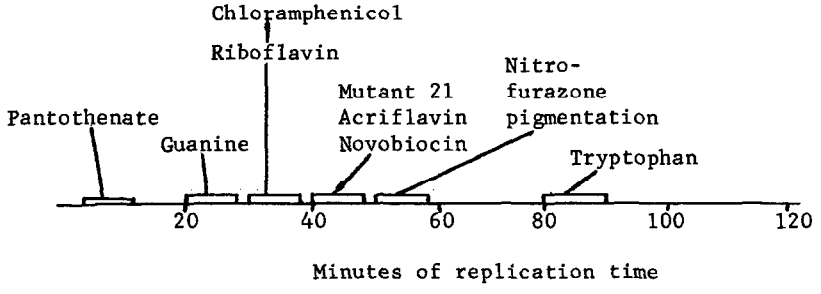
ACR = acriflavin resistance

NOV = novobiocin resistance

NFN = nitrofurazone resistance

CMP = chloramphenicol resistance

FIGURE 1. Chromosome map of Staph. aureus, strain Maybush.

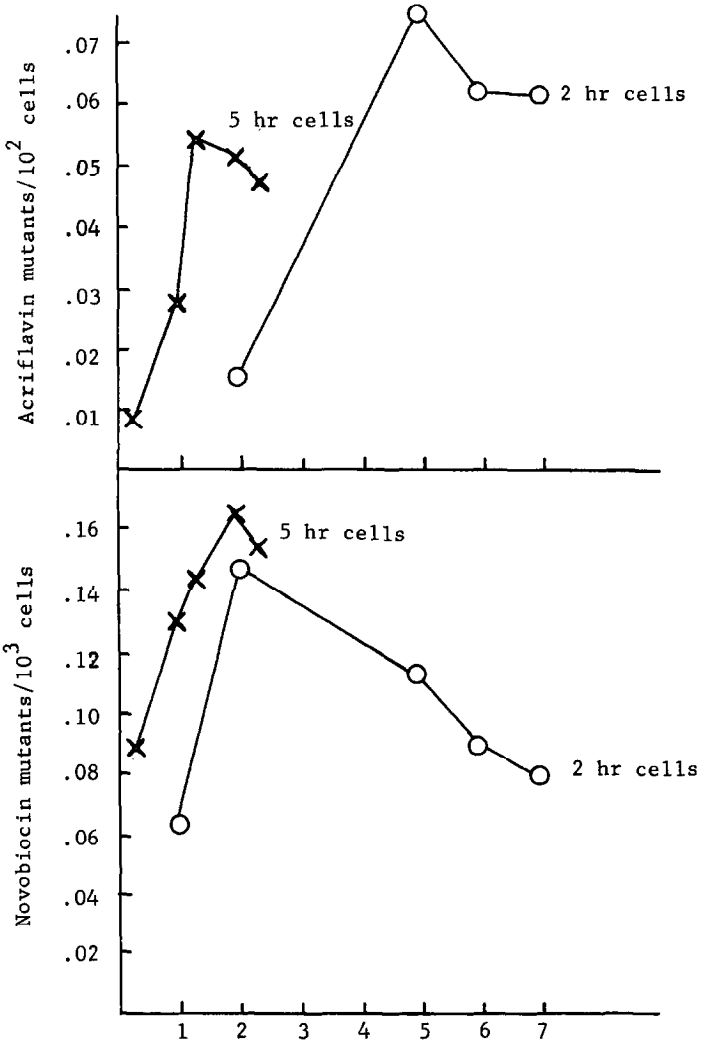


is compared to that of 5 hr cells. This marker behaves as a single locus with a chromosomal position defined in Fig. 1. The concept was then developed that acriflavin resistance locus occurred on a replicon separate from the replicon bearing the other genes presented in Table 1. It would be necessary for the replicon bearing acriflavin resistance (I) to be present in a greater number

of copies in early log phase (2 hr cultures) than the replicon (II) containing the other genes in Table 1. Furthermore, in mid to late log phase cultures (5 hr) the number of copies of replicon I relative to replicon II should decline several fold. This hypothesis would explain satisfactorily the shifting position of acriflavin resistance relative to the other genes as a function of culture age.

The hypothesis was tested by examining the kinetics of expression of acriflavin resistance as compared to expression of novobiocin resistance. Two hr and 5 hr cultures were prepared as indicated in the methods section. The 5 hr culture was diluted 1:10 in fresh medium so that both cultures possessed similar optical densities (0.05-0.06 at 650 mμ). Equal volumes (5 ml) of these cells were then centrifuged and resuspended in equal volumes of saline containing 200 μg of nitrosoguanidine/ml. After exposure for 20 min at 30 C, the cells were recovered by centrifugation and resuspended in 5 ml fresh, prewarmed (37 C) TS broth and placed on a shaker at 37 C. At appropriate intervals each culture was diluted and plated for total viability count and for numbers of induced mutants to acriflavin resistance and to novobiocin resistance (Fig. 2). With 2 hr cells, the expression of acriflavin resistance is significantly delayed as compared to expression of novobiocin resistance. In contrast, with 5 hr cells, the full expression of resistance to acriflavin occurs simultaneously with expression of novobiocin resistance. The ratio of frequency of acriflavin resistant mutants to frequency of novobiocin resistant mutants is 2.5 times higher with the 2 hr cells than with the 5 hr cells. These data are entirely consistent with the hypothesis that at 2 hr, there are many more copies of replicon I than of replicon II, resulting in a long segregation lag for expression of acriflavin resistance as compared to novobiocin resistance and yielding a much greater relative number of acriflavin resistant mutants than novobiocin resistant mutants. In 5 hr cells, where hypothetically, duplication of replicon I relative to replicon II has diminished, similar expression kinetics for acriflavin resistance and for novobiocin resistance would be expected.

FIGURE 2. Expression of mutations to acriflavin resistance and to novobiocin resistance in 2 hr and 5 hr cells after exposure to nitrosoguanidine.



Although the experiments outlined above could be applied to the specific case where the acriflavin locus duplicated at the same time as the novobiocin locus on the genomic map, the argument loses its validity when directed toward a locus which duplicates earlier than novobiocin resistance.

Therefore a technique was adopted whereby the numerical ratios of the number

of copies of replicon I relative to replicon II could be "frozen". This was done by addition of 0.40% phenylethyl alcohol (PEA) to the cultures and allowing subsequent incubation at 30 C for 2 hr. Cells so treated would be held invariant regarding the number of copies of both replicon I and II. Cultures of the wild type and the various auxotrophic mutants were grown in TS broth for 2, 3, 4, and 5 hr and treated with PEA to allow replication in progress to proceed to completion. The 3, 4, and 5 hr cultures were diluted in TS broth containing 0.40% PEA to equal the optical density of the 2 hr culture (0.05-0.06). The cells from equal volumes of culture were then removed by centrifugation, exposed to nitrosoguanidine, recovered by centrifugation and resuspended in 5 ml of fresh TS broth. The resulting cultures were incubated on a shaker at 37 C for 5 hr to insure full expression of all mutations. The cells were then diluted and plated both on TS agar containing 2 μg of novobiocin/ml and on TS agar containing another appropriate inhibitor or on minimal agar to score for revertants to prototrophy. The novobiocin resistance locus was employed as a reference marker since general experience had shown that this gene could be consistently scored with high accuracy. The ratio of the number of mutants at a specific locus relative to the number of novobiocin resistant mutants was calculated for the 2, 3, 4, and 5 hr cells. The results of four such determinations are presented in Fig. 3. The ratio of mutants at the pantothenate locus or the acriflavin resistance locus compared to novobiocin resistance mutants is high for the 2 hr cells but declines rapidly for the 3, 4, and 5 hr cells. The ratios of mutants at both the tryptophan locus and the nitrofurazone resistance locus relative to novobiocin resistant mutants are essentially constant. On the basis of such data, acriflavin resistance and pantothenate loci are assigned to replicon I and novobiocin resistance and tryptophan loci can be assigned to replicon II. The pantothenateless mutant grows noticeably more slowly in TS broth than most of the other mutants or the wild type so that the decline in ratio for pantothenate to novobiocin is less precipitous than for acriflavin to novobiocin. In Table 2 are the ratios of the frequency mutants at a variety of loci (Fig. 1) compared

FIGURE 3. Ratios of mutations of several loci to mutations to novobiocin resistance in 2, 3, 4, and 5 hr cells.

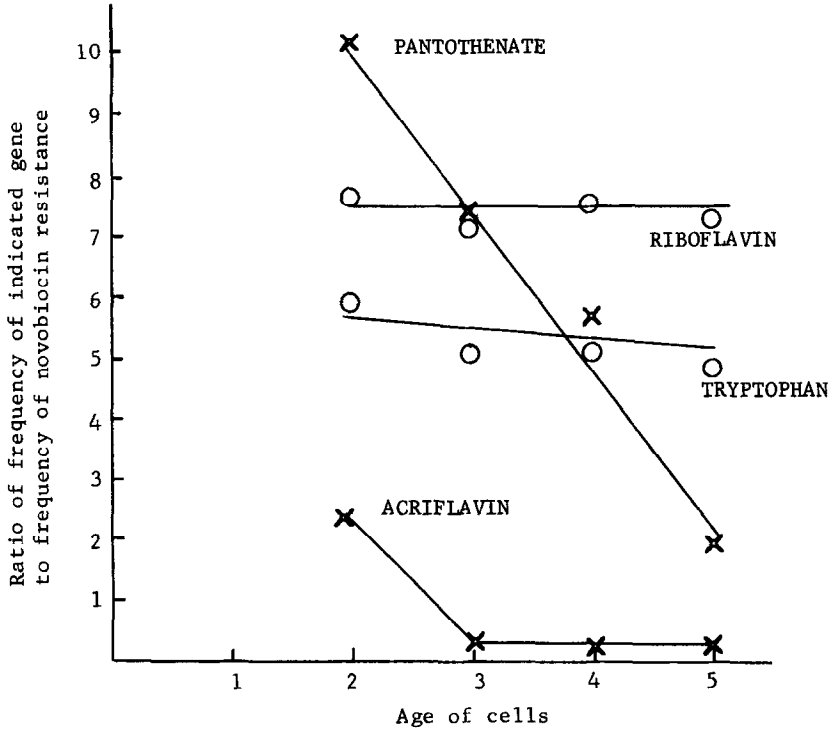


TABLE 2. Ratios of frequency of single genetic loci to novobiocin resistance locus as related to age of cells.

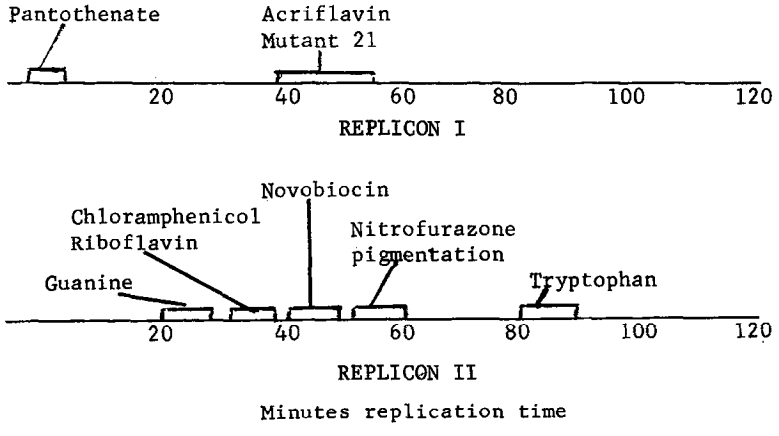
	Culture age			
	2 hr	3 hr	4 hr	5 hr
ACR	2.2	0.35	0.25	0.41
NFN	2.1	2.5	1.8	2.6
CMP	1.9	1.5	1.3	1.7
PAN	10.0	7.4	5.7	2.0
RIB	0.77	0.72	0.75	0.67
GUA	0.04	0.024	0.027	0.023
TRY	6.0	5.1	5.1	4.8
MUT 21	5.1	1.3	0.6	0.6

All values given are the relative gene frequencies in a population of cells but they are not normalized to the values of reference cells.

to the frequency of novobiocin resistant mutants. The two types of relationship as depicted in Fig. 3 can be clearly detected. Mutant 21 is an auxotrophic mutation, not yet defined, which has a gene duplication time of 40-50 min on the 120 min map (Fig. 1).

In Fig. 4 are presented the two hypothetical replicons bearing the respective genes at the duplication times determined by the synchronous chromosomal replication method. The distribution of nutritional and inhibitor resistance genes appears to be random. No rational conclusion can be drawn concerning the absolute numbers of replicons per cell. The ratios tell only that at 2 hr, replicon I is 5.1 (mut 21) to 6.4 (pan) times as frequent as replicon II when compared to the ratio of frequencies in the 5 hr cells. This concept also explains an anomaly in gene frequency analysis concerning the normalized value of

FIGURE 4. Two hypothetical replicons in Staph. aureus



Pigmentation gene exhibits full expression of mutation after two generations for both 2 hr and 5 hr cells.

pantothenate gene to novobiocin gene which value was 12.0 or more in several determinations and appeared to be in error for unknown reasons.

No conclusions can be drawn concerning the relative size of the two replicons proposed for Staph. aureus. They are approximately of equivalent length since replicon I bears a gene (acriflavin resistance) which duplicates at 40-50 min on the 120 min map whereas replicon II has only two later genes: nitrofura-

zone resistance (50-60 min) and tryptophan (80-90 min).

In conclusion, this rationale and methodology have not been applied to other organisms. To the author's knowledge there are no recorded cases of shifting gene order by gene frequency analysis in other bacteria as a function of the age of the cells. Although the concept of more than one replicon constituting the genome of a bacterium is novel, it is invariably true in higher organisms with visible chromosomes.

REFERENCES

Altenbern, R. A., Biochem. Biophys. Res. Communs. 25:346 (1966).
Jacob, F. and E. L. Wollman, Sexuality and the genetics of bacteria. Academic Press, Inc., New York (1961).
Treick, R. W. and W. A. Konetzka, J. Bacteriol. 88:1580 (1964).
Yoshikawa, H., and N. Sueoka, Proc. Natl. Acad. Sci. U.S. 49:559 (1963).