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Chromosome Mapping in *Staphylococcus aureus*

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The genome of *Staphylococcus aureus* was mapped by enumerating mutants induced by nitrosoguanidine during synchronous chromosomal replication following release from phenethanol inhibition. Both chromosomal replication time and cell division time were 120 min for this strain of *S. aureus*. Duplication of genes occurred within a 10-min period of the 120 min required for chromosomal replication. A high-resolution method was devised to determine the gene order of four genes that duplicated in the same 10-min interval of replication of the chromosome. A genomic map locating the positions of 10 genes was derived.

The present paper is an extension of work briefly reported previously (1). Chromosomal mapping of bacteria displaying no sexuality has been very limited. Stonehill and Hutchison (4) recorded a method for mapping several genes of *Streptococcus faecalis* by synchronizing chromosomal replication by amino acid starvation followed by periodic exposure of cells to ultraviolet light after resupplementation of the required amino acid.

A gene frequency analysis method for mapping the genome of transformable strains of *Bacillus subtilis* was introduced by Yoshikawa and Sueoka (5).

The data presented here show that the chromosome of *Staphylococcus aureus* can be mapped by periodic nitrosoguanidine treatment of cells undergoing synchronous chromosomal replication after release from phenethanol inhibition. These results, considerably refined compared to the original publication (1), demonstrate the reliability of the procedure and fully support the concepts leading to the development of this method of chromosomal mapping.

MATERIALS AND METHODS

The strain of *S. aureus* employed was isolated from clinical material submitted for analysis to the laboratory of the Frederick Memorial Hospital, Frederick, Md. It was maintained on BBL Trypticase Soy Agar (TSA) slants stored at 4 C and transferred every 3 months. Phenethanol was obtained from Eastman Organic Chemicals, Rochester, N.Y., and was filter-sterilized before use. Nitrosoguanidine was purchased from the Aldrich Chemical Co., Milwaukee, Wis. Solutions of nitrosoguanidine were prepared fresh daily and sterilized by filtration just before use.

Synchronous chromosomal replication procedure. To 100 ml of fresh BBL Trypticase Soy Broth (TSB), 10 ml of an 18-hr culture of an auxotrophic mutant or the wild type of *S. aureus* in TSB was added. This culture was incubated on a shaker for 3 hr at 37 C. Phenethanol was then added in a final concentration of 0.40% and the culture was incubated without shaking for 2 hr at 30 C. It was essential that the culture be agitated for several minutes to insure complete solution of phenethanol. The cessation of deoxyribonucleic acid (DNA) synthesis during such treatment is illustrated in Fig. 1a. A control culture showed considerably more DNA synthesis that stopped after about 75 min of incubation (Fig. 1a). After this incubation, 40 ml of the culture was centrifuged and the cells were suspended in 100 ml of fresh, one-half strength TSB prewarmed to 30 C. This culture was then incubated at 30 C. The immediate resumption of DNA synthesis after release from phenethanol inhibition is presented in Fig. 1b. The synthesis of DNA progressed at a linear rate up to 120 min of incubation, at which time there was an abrupt rate change equaling approximately a doubling of the original rate. Total viable counts during incubation after release from phenethanol inhibition yielded the results presented in Fig. 1c. It is clear that the cell division time is identical to the chromosomal replication time, as judged by the constant rate of DNA synthesis over the first 120 min of incubation. Lark (2) has shown that, for *Escherichia coli*, the cell division time and the chromosomal time are nearly identical until the cell division time becomes very long. At zero-time and every 10 min thereafter, a 5-ml sample of the cell suspension in one-half strength TSB was withdrawn, and the cells were recovered by centrifugation and resuspended in 5 ml of sterile saline containing 200 μg of nitrosoguanidine per ml and incubated for 20 min at 30 C. The suspension was then either diluted in saline and plated on minimal medium to detect induced prototrophs or the cells were recovered by centrifugation and resuspended in ice-cold TSB. The cold cell suspensions were quickly warmed to 37 C and incubated on a shaker at 37 C for 3 hr to permit expression of inhibitor resistance. The cells were then diluted and plated on TSA containing the proper concentration of inhibitor. Prepara-

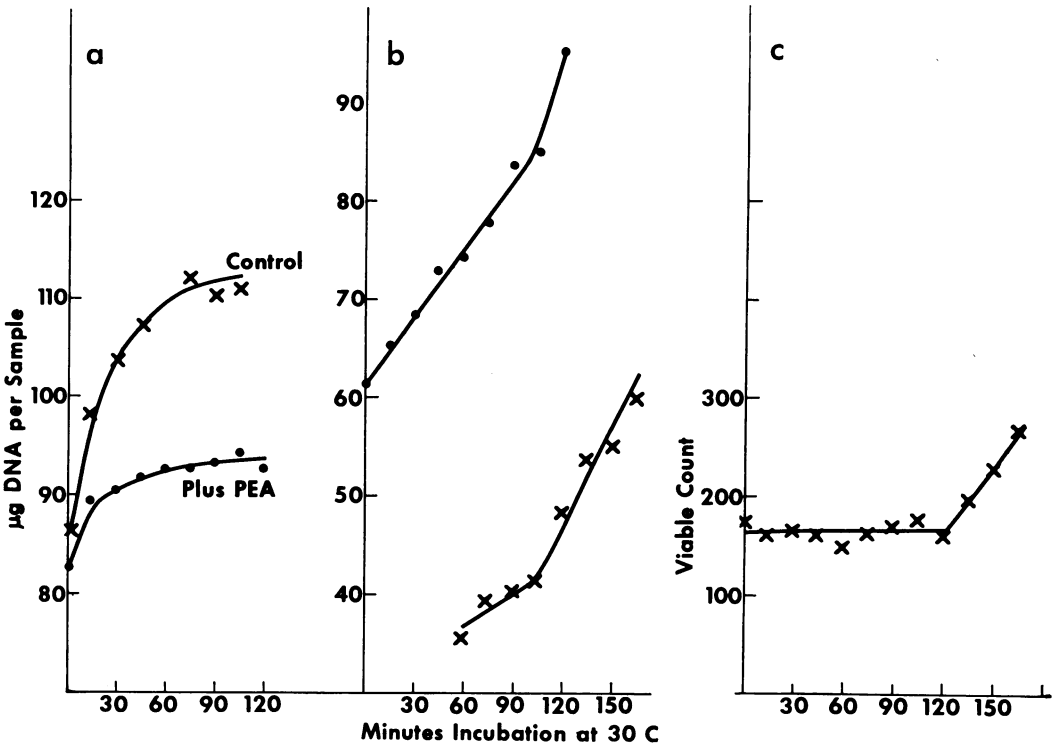

FIG. 1. *DNA synthesis and viable count during synchronous replication procedure. (a) Synthesis of DNA (μg/5 ml of culture) after addition of phenethanol to 3-hr culture; all points are averages of duplicate determinations. (b) Synthesis of DNA in one-half strength TSB after removal of phenethanol; all points are averages of duplicate determinations. Upper curve shows DNA synthesis (μg/8 ml of culture) for the first 120 min of incubation; lower curve shows DNA synthesis (μg/5 ml of culture) during the period of from 60 to 150 min after removal of phenethanol and shows the doubling of rate of DNA synthesis at 120 min. (c) Viable count in one-half strength TSB after release from phenethanol inhibition; all counts are total colonies on triplicate plates of 10^{-6} dilution. DNA analysis: cells from 5 or 8 ml of culture were sedimented by centrifugation and extracted twice with 5-ml portions of cold 5% trichloroacetic acid. Cells were then extracted with 2.5 ml of 5% acid at 90 C for 10 min. The suspension was centrifuged and DNA in the supernatant fluid was determined colorimetrically by the diphenylamine reaction (Burton reaction).*

tion of the minimal medium and other details of the procedure can be found in an earlier paper (1).

High-resolution modification. A culture undergoing synchronous chromosomal replication was prepared as described above. After 45 min of incubation and every 2 or 4 min up to 71 min of incubation, 4 ml of culture was withdrawn and added to 1 ml of saline containing 1 mg of nitrosoguanidine. These suspensions were incubated at 30 C for exactly 20 min and then diluted 1:10 in ice-cold saline. The cells were then collected by centrifugation and resuspended in 1 ml of fresh TSB. These cultures were incubated, with shaking, at 37 C for 6 hr and then diluted and plated on TSA containing the appropriate inhibitor.

The data presented in Fig. 2 and 4 represent the total mutant count at any time on triplicate plates spread with 0.1 ml of the same dilution of the treated cells. The inhibitor concentrations in the selective media were as follows: chloramphenicol, 4 μg/ml; novobiocin, 2 μg/ml; nitrofurazone, 8 μg/ml; acriflavine, 25 μg/ml; and vancomycin, 2 μg/ml.

RESULTS

Some representative data for determining gene duplication time are presented in Fig. 2. The number of mutants induced in the exposed cells was constant for an initial period, followed by a sharp increase by a factor of approximately 2.0. The sharp increase in mutant numbers occurred while the total viable count was constant (Fig. 1c). The time at which the mutant count changed is designated as the replication time of the gene in question. Judging from the abrupt change in mutant numbers obtained, chromosomal replication was well synchronized. The sharp increase in numbers of mutants at any particular locus occurred over a 10-min incubation period (one-twelfth of the chromosomal replication time). Therefore, the gene loci can be assigned to a certain 10-min segment of the total chromosome. The reliability of the method is well illustrated by

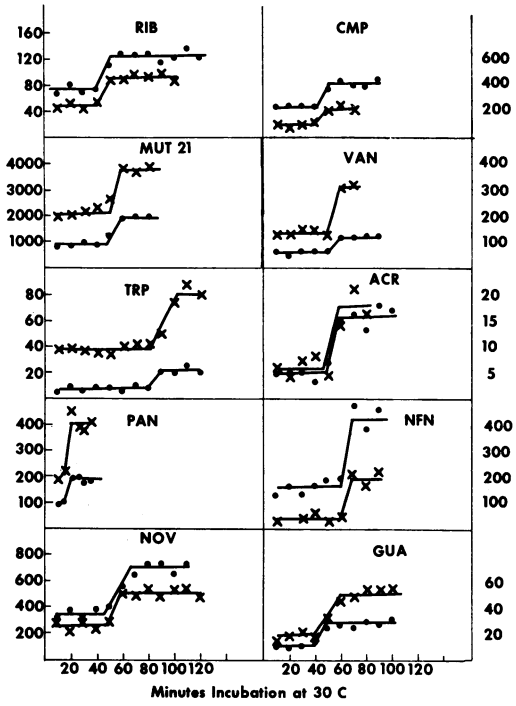

FIG. 2. *Increase in mutant numbers at 10 loci during synchronous chromosomal replication. Values on the ordinates are total mutant counts on triplicate or sextuplicate plates of the same dilution. Plates incubated for 48 to 72 hr at 37 C. Higher plate counts in replicate experiments result from greater numbers of plates or greater incubation time.*

TABLE 1. *Ratio of average number of mutants after increase to the average number of mutants before increase*

Gene[a]	Single experiments	Avg of experiments
PAN	1.78, 1.74	1.76
RIB	1.67, 1.85	1.76
NOV	1.85, 1.90, 1.97, 1.80	1.88
ACR	2.45, 2.40, 2.69	2.51
VAN	1.93, 2.40	2.16
TRP	2.13, 2.27	2.20
CMP	1.71, 2.02, 2.45, 2.27, 1.90	2.07
NFN	7.90, 4.40, 2.53	4.71
MUT-21	1.76, 2.02	1.89
GUA	2.70, 2.30	2.50

[a] Abbreviations: *pan*, pantothenic acid; *rib*, riboflavine; *nov*, novobiocin; *acr*, acriflavine; *van*, vancomycin; *trp*, tryptophan; *cmp*, chloramphenicol; *nfn*, nitrofurazone; *mut*, undiagnosed nutritional mutant; *gua*, guanine. Anomalous behavior of *nfn* locus is due to much greater than twofold increase in resistant clones when a twofold greater number of resistant cells is plated.

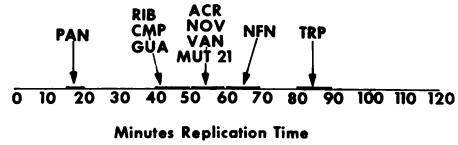

FIG. 3. *Gene locations determined by mapping procedure. Abbreviations are defined in Table 1.*

the data in Fig. 2, which presents the results of two independent experiments for each gene examined. The average mutant count after the abrupt increase, divided by the average count before the increase, yields a value reasonably close to 2.0, the theoretical value. The actual values of this ratio are presented in Table 1 for a number of experiments. Such data are entirely consonant with a doubling of the genes under consideration. Coupled with the demonstration of a linear rate of synthesis of DNA after release from phenethanol inhibition, these results strongly indicate that there is only a doubling of the chromosome during the 120-min incubation period and that there is only one growing point during chromosomal replication under these conditions. The genomic map obtained from the foregoing data is presented in Fig. 3. As previously stated, each gene is assigned to a certain 10-min segment within which duplication has occurred. Since the centrifugation time necessary to sediment the cells for each sample was 10 min, chromosomal replication had proceeded for that time period before the cells were exposed to nitrosoguanidine. Therefore, the results for the zero-time sample were plotted at points 10 min later than the time of withdrawal of the sample from the main culture.

Five genes mapped in the 50- to 60-min segment (Fig. 3). Application of the high-resolution method to determine the order of duplication of the four inhibitor resistance genes in this segment yielded the results presented in Fig. 4. The novobiocin and vancomycin resistance genes duplicated first and are probably indistinguishable, followed by acriflavine and nitrofurazone resistance genes in that order. The rate of increase in numbers of mutants appears less abrupt by this procedure because the samplings are so frequent. The high-resolution method involves exposure of cells in growth medium to nitrosoguanidine as compared to exposure in saline by the other procedure, so that the duplication times of the genes by the two methods do not show an exact match. Nevertheless, a sequence of these genes can be obtained by the high-resolution

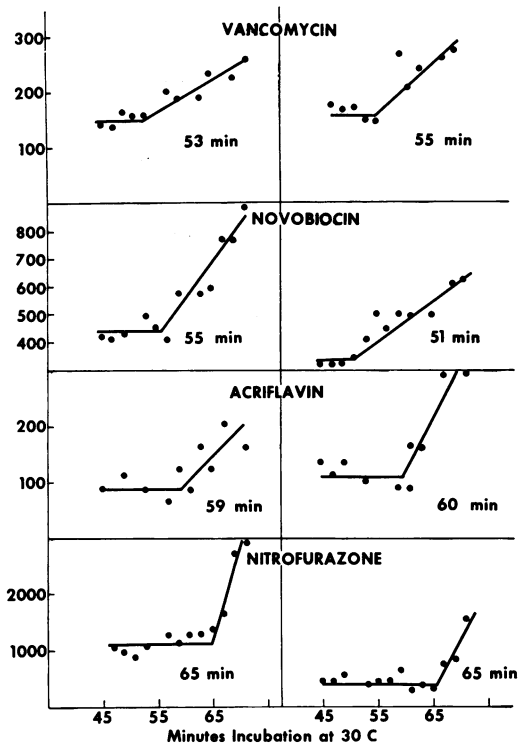

FIG. 4. *High-resolution mapping of loci determining resistance to novobiocin, acriflavine, nitrofurazone, and vancomycin. Values on the ordinates are total mutant counts on triplicate plates of the same dilution.*

method which has been confirmed by gene frequency analysis (*unpublished data*).

DISCUSSION

The results presented here demonstrate that genomic mapping of *S. aureus* can be accomplished by synchronizing chromosomal replication with phenethanol treatment followed by periodic exposure of cells to nitrosoguanidine. The much higher degree of synchrony obtained with this procedure, resulting in 120-min chromosomal replication time as compared to the 60-min replication time previously published (1), is difficult to explain, but apparently nutritional conditions following release from the phenethanol block exert considerable influence on phasing of chromosomal replication. It is clear that only duplication of the genes occurred. There was no evidence that more than two copies of the gene arose during chromosomal replication.

There has been no direct demonstration here that *S. aureus* cells exposed to phenethanol have completed, resting chromosomes. However, the immediate resumption of DNA synthesis during incubation after release from phenethanol inhibition, and the reliable apparent gene duplication at predictable times during this incubation, make the assumption of synchronous chromosomal replication a reasonable one.

Under the influence of phenethanol, all chromosomes cease replication, apparently at the same point, since after removal of phenethanol they reinitiate replication at the same point. Tentatively, these two points can be designated as the terminus and the origin, respectively, of chromosomal replication.

Phenethanol clearly inhibits DNA synthesis under the present conditions within 45 min of its addition to the culture. The increase in total DNA amounts to 1.1 to 1.2 times the amount present at the time of addition of phenethanol. This value is somewhat less than that expected if all stages of chromosomal replication were random in the cell population. On this basis, it is proposed that most of the chromosomes were in the later stages of duplication at the time of addition of phenethanol. Since the cultures were in late log to early stationary phase at this time, such an assumption does not seem unreasonable.

After release from phenethanol inhibition, the total DNA after 120 min of incubation (the cell division time) is only 1.5 times the amount present at the moment of release of inhibition. This phenomenon is consistent for the wild type and has also been observed for the auxotrophs employed (1). It is known that there is no loss in viability throughout the incubation in the presence of phenethanol (*unpublished data*). The failure of the DNA amount to double by cell division time has no ready explanation except for the possible existence of two chromosomes per cell, only one of which duplicates after release from phenethanol inhibition (2).

While the high-resolution mapping method described here was able to show the sequence of four genes, all duplicating in a 10-min period of chromosomal replication, the results made it clear that no greater resolution could be obtained by further refinements in the procedure. However, the general method proposed in this investigation can readily reveal genes relatively close to each other, thereby leading to possible fine-structure determination of the order of linked loci by transduction.

After this investigation was completed, my attention was directed to a paper by Ryan and Cetrulo (3), who showed that, in synchronized populations of *E. coli*, mutations induced by 2-amino purine occurred only during replication of the locus in question. These authors fully recognized the possible applicability of their

results to a system for chromosomal mapping similar to the method described in this paper.

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