APPARENT GENOMIC MAPPING OF STAPHYLOCOCUS AUREUS BY A NEW METHOD

ROBERT A. ALTENBERN

Biological Sciences Laboratory, Fort Detrick, Frederick, Maryland

Received October 17, 1966

The classical methods for mapping bacterial genomes are dependent upon matings between male and female pairs and are thus restricted to those genera exhibiting sexuality. Subsequently Sueoka (1965) devised a method of mapping involving transformation of auxotrophic Bacillus subtilis by DNA extracted during synchronous chromosomal replication of the wild type. These methods, however, rely on some type of gene exchange and can be applied only to those organisms than can undergo gene transfer.

The data in this communication indicate that the chromosome of Staphylococcus aureus can be mapped by enumerating mutants induced by exposure to nitrosoguanidine at various times during synchronous DNA replication. Theoretically this method should be applicable to any bacterium in which chromosomal duplication can be synchronized.

Mapping procedure: Ten ml of an 18-hr culture of an auxotrophic or wild type clone of Staph. aureus in Trypticase Soy broth (Baltimore Biological Laboratories) were added to 100 ml of fresh Trypticase Soy (TS) broth. This culture was incubated for 3 hr at 37 C in a shaker, at which time it was entering the stationary phase. Phenethyl alcohol was added in a final concentration of 0.4%, which allowed a 1.4 to 1.5 increase in total DNA followed by complete inhibition of further DNA synthesis. Following the procedure of Treick and Konetzka (1964) for Escherichia coli, the cells were then incubated in the presence of phenethyl alcohol for 2 hr at 30 C without shaking.

At this point all cells have completed chromosomal replication but are unable to begin a new round of replication, presumably by preventing synthesis of initiator. After this incubation period, 40 ml of the culture was centrifuged and the cells were resuspended in 100 ml of fresh TS broth prewarmed to 30 C, thus releasing the cells from phenethanol inhibition and allowing chromosomal replication to commence. This cell suspension was then incubated without shaking at 30 C. At zero time and every 10 min thereafter a sample was withdrawn and the cells were quickly sedimented by centrifugation and resuspended in a sterile solution of 200 μg of nitrosoguanidine per ml in saline. This suspension was then incubated at 30 C for 20 min, following which the cells were either diluted and plated on minimal medium to detect mutations to prototrophy or centrifuged and resuspended in ice-cold TS broth. The cold cell suspensions were warmed quickly in a 37 C water bath and incubated on a shaker at 37 C for 3 hr to allow for expression of inhibitor-resistant mutants. The cells were then diluted and plated on TS agar containing an appropriate concentration of the inhibitor. All plates were incubated at 37 C until the colonies had attained countable size. Mutations to prototrophy of some auxotrophs required longer incubation periods than others to obtain satisfactory colony size. The crowding phenomenon was severe in the case of some mutants and numbers of mutant colonies had to be determined on plates of the same dilution to give meaningful results.

All auxotrophic mutants were isolated from suspensions of the wild type exposed to nitrosoguanidine. Mutagen-treated cells were plated either on TS agar or on minimal agar supplemented with a single growth factor. Replica plating to minimal agar allowed detection of the induced auxotrophs. Minimal agar in this case had the following ingredients per liter: acid-hydrolysed casein (vitamin free) - 15 g, ammonium sulfate - 2 g, K_2HPO_4 - 14 g, KH_2PO_4 6.0 g, $MgSO_4$ - 0.2 g, thiamine HCl - 10 mg, niacin amide - 10 mg, glucose (added aseptically) - 5 g, and agar - 15 g. Single auxotrophs requiring riboflavin, pantothenate, guanine, or tryptophan have been isolated to date.

The DNA content of cells was determined on the hot TCA extract employing the Burton reagents. Optical density of cultures was determined with a Coleman Junior Spectrophotometer at 650 mμ. Phenethyl alcohol was filter-sterilized before use. Nitrosoguanidine solutions were prepared and filter-sterilized just before use because of the marked instability of this compound in solution.

Results: Preliminary experiments showed that the maximum numbers of prototrophs were induced in a saline suspension of auxotrophs by exposure to 200 μg of nitrosoguanidine per ml for 20 min. Longer exposure times or higher mutagen concentrations resulted in progressively fewer prototrophic mutants, probably because of the pronounced killing effect of the mutagen. Contrary to the slight killing of E. coli exposed to nitrosoguanidine in a non-growth medium (Adelberg et al., 1965), Staph. aureus suffered approximately a 2-log drop in survivors following the mutagen exposure described above.

A critical point for establishing the validity of the mapping method described here rested upon the demonstration that the total number of mutants induced at a particular locus by nitrosoguanidine was a direct and measurable function of the amount of DNA present. Suspensions of the riboflavinless mutant were prepared so that one suspension (2 X) contained twice the number of cells as the other (1 X). Quadruplicate samples of these suspensions were centrifuged and the cells resuspended in nitrosoguanidine (200 μg/ml in saline) and exposed for 20 min at 30 C. They were then diluted and plated on minimal agar and the numbers of prototrophic mutants were determined after suitable incubation. The results in Table 1 show that there were four times as many mutants in the survivors from the 2 X suspension as the 1 X suspension. Since there were twice as many surviving bacteria in the 2 X suspension, these results show that, with a twofold increase in DNA, there is a concomitant twofold increase in mutations at a particular locus when treated with a constant concentration of nitrosoguanidine for a prescribed time.

Consequently, after any particular gene has duplicated during synchron-

ous chromosomal replication, exposure to nitrosoguanidine should yield twice as many mutants at this locus than arise from mutagen treatment before duplication of the gene.

TABLE 1. Yield of prototrophs following exposure of riboflavinless mutant of Staph. aureus to nitrosoguanidine.

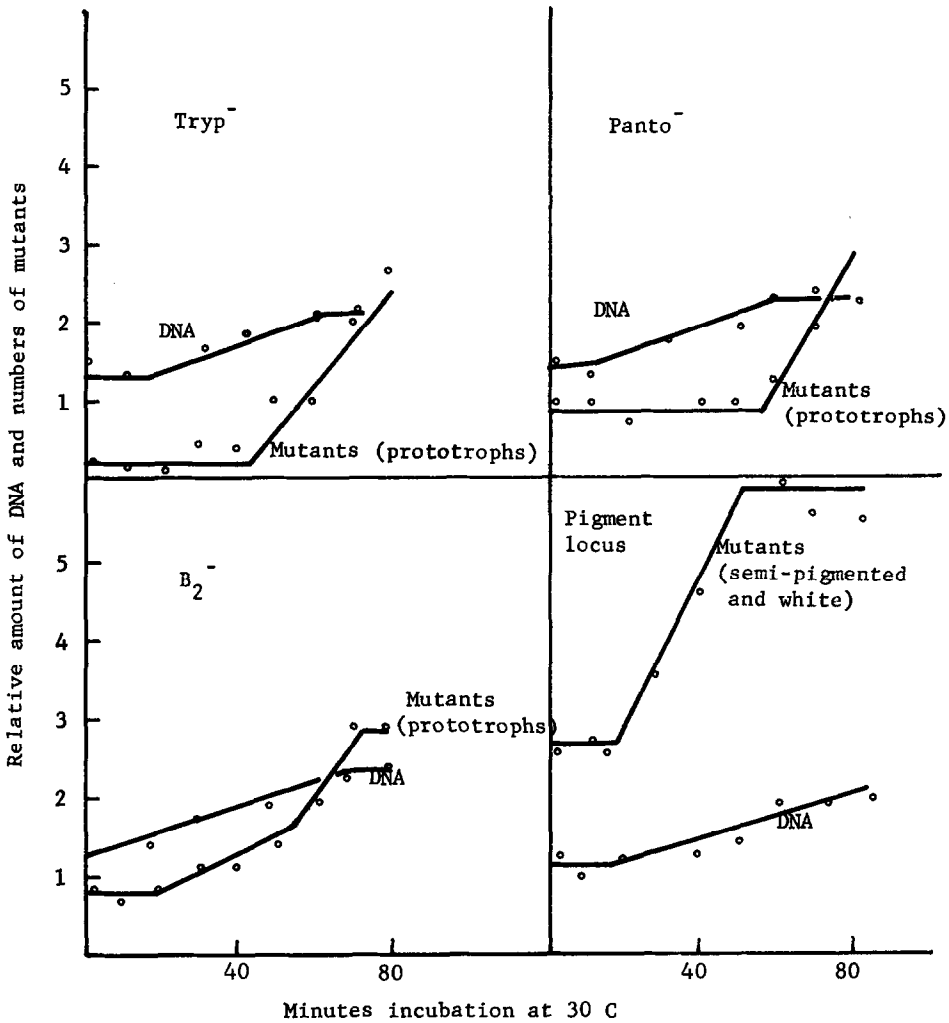
Cell concentration	Average no. of mutants*	Ratio 2 X/1 X
1 X	20.5	
2 X	85.0	4.1

* Average number of prototrophs per plate on 12 plates of 10^{-1} dilution of treated cell suspension.

Mutants singly auxotrophic for riboflavin, pantothenate, or tryptophan were subjected to the mapping procedure. The numbers of mutations to prototrophy and the DNA synthesis curves are presented in Fig. 1. Included in this figure are the results of mapping the wild type for the pigment locus in which mutations to white were counted. It is clear that an increase in numbers of mutants at the loci investigated occur at different and distinctive times during DNA synthesis. The replication time of the locus in question has been determined by the intersection of the line where mutant number is constant with the line showing the increase in mutant numbers. Repeat experiments have shown that replication time for a locus is reproducible to within 5 min.

The slope of increase in mutations suggests that, although chromosomal replication may be roughly synchronized, chromosomes of individual cells may replicate at slightly different rates.

As judged by the DNA analysis, chromosomal replication requires approximately 60 min under these experimental conditions. When the DNA content of the cells reaches an apparent plateau at 60 min there is only 1.4 to 1.5 times as much DNA as at zero time. The reasons for this lack of complete doubling of DNA content are not clear but it presumably results either from a possible 50% loss in viability during phenethanol treatment or from replication of only one of two possible chromosomes per cell as a consequence of the



	At zero time	
	μg DNA/ml culture	Total mutants on triplicate plates
Tryp⁻	28	1
Panto⁻	29	16
B_2^-	13	83
Pigmented parent	45	52

FIGURE 1. Number of mutations of four loci during chromosomal replication of Staph. aureus.

shift down in temperature from 37 C to 30 C as noted in the methods. Early trials showed that chromosomal replication following release from phenethanol inhibition at 37 C was too rapid for accurate mapping.

Determination of both the growth rate by turbidity and the total viable cells in a population of the riboflavinless mutant subjected to the mapping procedure yielded the results presented in Fig. 2. There is no significant increase in cell numbers (no division) until after 60 min of incubation. The growth rate is very low, giving a mass duplication time of nearly 5 hr.

The secondary rise in mutant numbers noted at 70 and 80 min in many mapping experiments reflects only cell doubling and has no significance in regard to genomic mapping.

Both the riboflavinless and the guanineless mutants were subjected to the mapping procedure and the survivors were scored both for mutations to prototrophy and to antibiotic resistance. The results (Fig. 3) show clearly that two loci map at distinctly different times when the replication times of both are determined in the same cell suspension. These data and the observations on total viable cells in the population during the mapping procedure rule out the following two possible explanations for the data so far

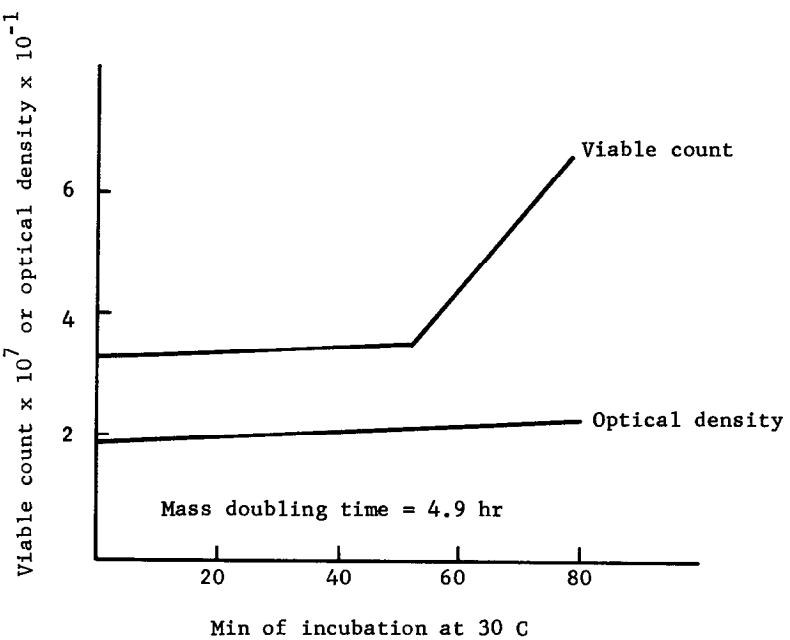


FIGURE 2. Mass increase and total cell numbers of riboflavinless mutant of Staph. aureus during mapping procedure.

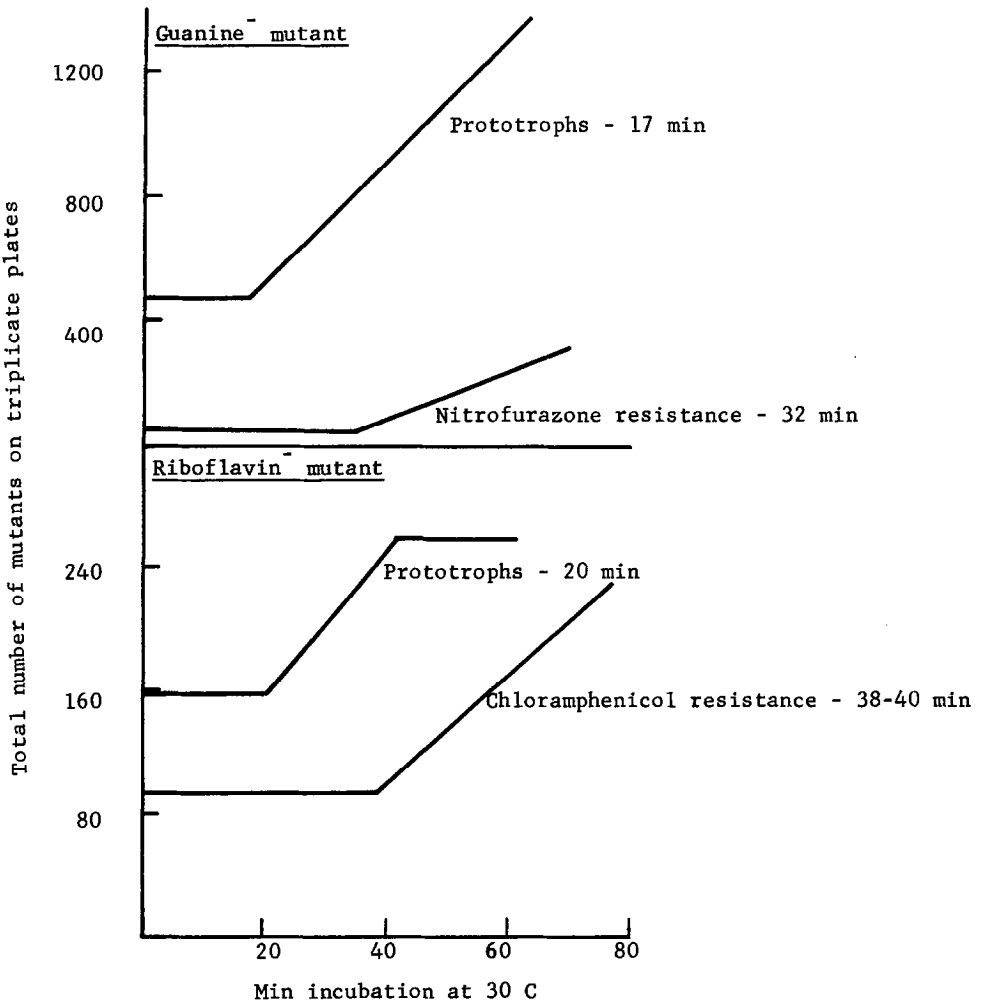


FIGURE 3. Determination of gene duplication time of two loci during chromosomal replication.

collected; (1) each auxotrophic mutant divides at a characteristic time after release from phenethanol inhibition, yielding a twofold increase in mutant numbers owing to a doubling in cell number and (2) at a specific time after release from PEA, a nonspecific increase in general mutability occurs.

The total number of loci mapped to date is presented in Fig. 4, based on a 60-min chromosomal replication time. Further additions to the map are severely limited at present because of the difficulty in obtaining auxotrophs, although resistance to various antimicrobial substances can be readily mapped.

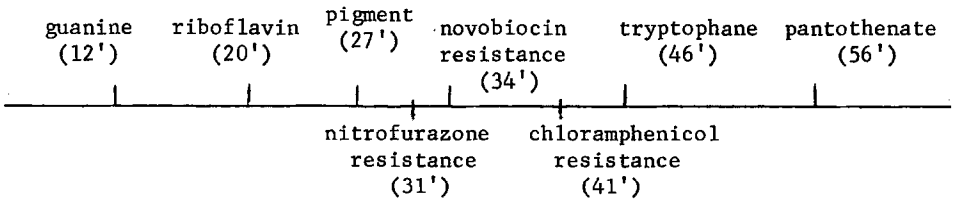


FIGURE 4. Preliminary map of the chromosome of Staph. aureus. Total length represents 60 min.

Although this novel mapping method has not yet been tested with other organisms, there seems to be no fundamental reason why the technique cannot be applied to any bacterium, provided that chromosomal replication can be synchronized and that treatment with nitrosoguanidine induces a significant number of mutants at any given locus. Adjustment of total chromosomal replication time to 120 min should give a method of genomic mapping with resolution equivalent to that obtained by interrupted mating techniques with E. coli. In addition, this method does not involve the tedious normalizing manipulations of the mapping technique described by Sueoka (1965).

References

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