*LINEAR SEQUENTIAL ARRANGEMENT OF GENES FOR THE BIOSYNTHETIC PATHWAY OF PROTOHEME IN STAPHYLOCOCCUS AUREUS**

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Staphylococcus aureus has the capacity to grow anaerobically in the presence of glucose without a functional electron transport system or without a complete tricarboxylic acid cycle.[1] In the presence of oxygen, a membrane-bound electron transport system is formed and the growth rate increases significantly.[2] When grown aerobically on nutrient agar where the principal carbon sources are amino acids, mutants that form the electron transport system very slowly can easily be differentiated from the wild type by the difference in colony size. The synthesis of protoheme (Fe protoporphyrin IX) is required for the formation of the heme enzymes of the electron transport system. Although the genetic analysis of the heme biosynthetic chain has been long sought in bacteria, it was not until the fortunate use of kanamycin on *Staphylococcus* that mutants in the protoheme biosynthetic chain could be readily prepared and tested for gene sequence by phage transduction. Some of the mutants are able to grow as rapidly as the wild type when 8 μM protoheme is added to the nutrient agar. The preparation of these mutants and their analysis by transduction is reported in this study.

Methods.—Strain U-71 of *S. aureus* and International Staphylococcal Typing phage 53 were supplied by J. W. Baldwin, University of Georgia. Bacteria were grown in nutrient agar or broth and trypticase soy agar or broth (Difco Products Corp.) at 37°C. The protoheme precursors: δ aminolevulinic acid (ALA), protoporphyrin, protoheme (Sigma Chemical Corp.), or porphobilinogen (PBG) (the gift of D. C. Mauzerall, Rockefeller University) were added at 8 μM where indicated in the text. Protoheme or protoporphyrin (20 mg) was dissolved in 0.5 ml pyridine, diluted with 0.5 *M* NH_4OH to 5 ml, and stored in the dark at −16°C. To select the mutants, a suspension of 10^7 cells from an 18-hr culture was plated on nutrient agar containing 6 μM kanamycin sulfate (Bristol Labs.). The plates were incubated 48 hr and the minute colonies transferred to protoheme-containing plates with sterile hardwood toothpicks. Mutants able to grow as rapidly as the wild type in the presence of protoheme were collected. Double mutants were selected by growing ALA auxotrophs with kanamycin and harvesting the small colonies from a nutrient agar plate containing ALA. The small-colony mutants from plates containing ALA, which grew normally in the presence of protoheme, were shown by transduction to have two lesions in the protoheme biosynthetic pathway. Transduction and phage propagation techniques were essentially those described by Pattee and Baldwin.[3] Briefly, the recipient culture was grown on nutrient agar with protoheme for 18 hr and suspended at a density of 10^{10} cells/ml in the presence of 1.5–2.0 $\times$ 10^{10} phage/ml for 30 min at 37°C. The mixture was then placed in an ice bath, made to 11.3 mM with sodium citrate, and centrifuged. The pellet was resuspended in cold 67 mM citrate and a tenth of the volume plated on nutrient agar containing 0.19 mM sodium citrate. Controls for back mutation and phage sterility were included in each experiment. Bacteria for phage propagation were grown on trypticase soy agar for 12 hr and harvested. The bacteria were combined with 10^{10} phage in trypticase soy broth containing 0.5% agar and 4 mM $CaCl_2$ and poured on trypticase soy agar plates. The plates were incubated for 5 hr, the soft agar was scraped off, and washing was performed with three por-

tions of nutrient broth. The washing and soft agar scrapings were centrifuged, and the supernatant was filtered through Millipore filters of 0.45-μ pore size. Two cycles of phage growth were usually necessary to achieve a sufficiently high phage titer. The pigments of the electron transport system were assayed spectrophotometrically.[4] Oxygen utilization in the presence of L-lactate was measured polarigraphically.[4] Protoheme precursors were isolated, separated, and identified as described.[5]

Results and Discussion.—The nature of the mutants: Aerobic growth of *S. aureus* in nutrient agar involves the function of the electron transport system. When *S. aureus* is grown in nutrient agar containing kanamycin, dwarf-colony mutants are produced. After growth for 48 hours, the dwarf-colony mutants measure 0.1–0.5 mm and the wild-type colonies measure 2–4 mm in diameter. The dwarf colony mutants are unable to form the electron transport system as rapidly as the wild type. The doubling time of the mutants is 90–110 minutes compared to 50 minutes for the wild type in nutrient broth. The mutants are "leaky" in that they are able to grow slowly. In the presence of 50 mM glucose in nutrient broth, *S. aureus* does not form an electron transport system and growth is independent of oxygen. Both the mutants and the wild type grow at the same rate in the presence of glucose.

About 20 per cent of the mutants grow as rapidly as the wild type in the presence of 8 μM protoheme and in the absence of glucose. With protoheme these mutants form an electron transport system as rapidly as the wild type. The defect in the formation of the electron transport system in the protoheme-stimulated mutants is in the rate of protoheme synthesis. The protoheme is necessary for the formation of the cytochromes that are a part of the electron transport system.

Mutants stimulated by protoheme appear at a frequency of about 1 in 10^5 in the presence of kanamycin. The mutants revert to wild type at different rates ranging between 1 and 10 in 10^9 as determined by fluctuation tests. Phage grown on revertants transduce mutants as effectively as phage grown on the wild type. The mutants are neither resistant to nor dependent on kanamycin.

Chemical nature of the mutants: The pathway of protoheme biosynthesis involves the following reactions: (1) the condensation of pyridoxal-glycine and succinyl-coenzyme A to form ALA, (2) the condensation of 2 moles of ALA to form 1 mole of PBG, (3) the condensation of 4 moles of PBG to form uroporphyrinogen isomer III, (4) the sequential decarboxylation of the four acetyl-group side chains of the ring to form coproporphyrinogen III, (5) the oxidative decarboxylation of two propionic acid groups to form protoporphyrinogen, and (6) the chelation of iron by protoporphyrin to form heme.[6] Each step is catalyzed by a single enzyme except step 3, which requires the interaction of two enzymes.[6]

The protoheme-stimulated mutants were tested for the ability to grow rapidly in the presence of ALA, PBG, or protoheme in nutrient agar. Of 53 mutants, 15 grew rapidly in the presence of ALA, PBG, and protoheme and were considered to be mutants unable to form ALA. Eight mutants grew rapidly in the presence of PBG and protoheme but not with ALA and were considered to be unable to convert ALA to PBG. Thirty mutants grew rapidly if protoheme was added

to the nutrient agar, and these mutants were considered to be unable to convert uroporphyrinogen III to coproporphyrinogen III, coproporphyrinogen III to protoporphyrin IX, or protoporphyrin IX to protoheme. The porphyrinogens are unstable in air and could not be used to test for growth response. None of the mutants grew rapidly in the presence of protoporphyrin, which suggests that *S. aureus* is impermeable to protoporphyrin. Porphyrins have a distinctive red fluorescence when excited by ultraviolet light. Porphyrins are formed spontaneously from the porphyrinogens in the presence of oxygen and light. The mutants whose growth is stimulated by PBG or protoheme, when grown in presence of 8 μM PBG and 0.2 μM protoheme, form porphyrins which can be detected by the red fluorescence of the bacterial pellet after centrifugation. The 30 mutants that were able to grow rapidly in the presence of protoheme were grown with 8 μM ALA and 0.2 μM protoheme. The bacterial pellet had the characteristic red fluorescence of porphyrins. The porphyrins were extracted from the bacterial pellet with cyclohexanone and identified by the R_f values obtained, using ascending paper chromatography in a solvent of 2,4-lutidine:0.7 *M* ammonium hydroxide (10:7), v/v) as illustrated in Figure 1. Results of this analysis indicate that protoheme-stimulated mutants fall into three classes: (1) mutants unable to convert uroporphyrinogen III to coproporphyrinogen III (these form only eight carboxyl-containing porphyrins), (2) mutants unable to convert coproporphyrinogen III to protoporphyrin IX (these form porphyrins with four to eight carboxyl groups), and (3) mutants unable to convert protoporphyrin to protoheme (these form porphyrins with from two to eight carboxyl groups).

Experiments to demonstrate syntrophism (cross-feeding) indicate that the mutants able to grow rapidly with PBG are able to supply ALA to the ALA mutants. The porphyrins and presumably the porphyrinogens are bound very tightly to the cells and no porphyrin can be detected in the culture fluid after the bacteria have been removed by centrifugation. Syntrophism cannot be demonstrated between the porphyrinogen mutants.

Evidence from these experiments indicates that the 53 mutants involve five of the steps in protoheme biosynthesis. Each of the mutants appears to involve a single enzymatic step.

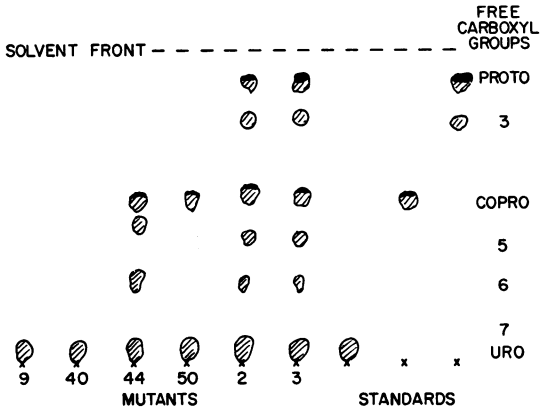

FIG. 1.—Ascending paper chromatogram of porphyrins isolated from the mutants grown with ALA and heme. Porphyrins were detected by their fluorescence in ultraviolet light after chromatography in a solvent of 2,4 lutidine : 0.7 *M* ammonium hydroxide (10:7, v/v).

Genetic analysis of the mutants: Two mutants chosen from each of the five classes of mutants were used as hosts for growth of transducing phage. The phage was then used for transduction with each of the pairs of mutants as recipients and the frequency of recombination to wild type determined (Table 1). Recombination frequencies between the mutants with lesions in the same biochemical step were 10^2 to 10^3 times less than recombination frequencies between mutants with defects in different biochemical steps. The efficiency of transduction was determined by the frequency of transduction to wild type with phage propagated on wild-type donors. The average recombination frequency for four mutants from each of the five classes of mutants was 1.4, 0.9, 1.7, 2.3, and $3.0/10^6$ phage for the ALA, PBG, copro, proto, and heme mutants.

Linkage: Mutants were selected that were unable to form ALA and unable to convert uroporphyrinogen to coproporphyrinogen (designated ALA-copro), and other mutants were selected that were unable to form ALA and unable to convert protoporphyrin to protoheme (designated ALA-heme). The transductional analysis of the double mutants is given in Table 2. These mutants were then used as recipients in transduction experiments with phage grown on wild-type hosts. The frequency of recombination to wild type per phage over a 100-fold range in multiplicity of infection remained relatively constant (Fig. 2). These data suggest that both genetic lesions are carried in the same phage.

Order of the genes: Results of transduction experiments between double mutants and various single mutants are given in Table 3. Assuming that a quadruple crossover is much less frequent than a double crossover, only one order is possible—namely, the order ALA-PBG-copro-heme. If phage, grown with an ALA mutant, is used as a donor for transduction with the double mutants of ALA-copro or ALA-heme as recipients, the frequency

TABLE 1. *Frequency of transduction to wild type between protoheme-stimulated mutants.*

Recipient	Donor								
	ALA-20	ALA-28	PBG-1	PBG-12	Copro-40	Copro-9	Proto-44	Proto-50	Heme-2	Heme-3
ALA-20	0*	10	310	1150	990	1510	1750	1550	1610	2450
ALA-28	20	0	140	920	805	875	1295	1190	1240	1495
PBG-1	160	60	0	0	290	390	590	740	880	1210
PBG-12	270	95	25	0	315	365	530	530	670	665
Copro-40	570	440	540	520	0	20	130	305	220	245
Copro-9	490	370	250	195	40	0	95	325	350	195
Proto-44	730	540	550	330	110	20	0	10	40	80
Proto-50	780	635	610	415	125	90	0	0	75	180
Heme-2	1070	840	1525	645	170	345	150	75	0	45
Heme-10	1200	1100	1010	550	320	410	210	240	20	10

* Wild-type recombinants/10^9 phage scored after growth in nutrient agar.

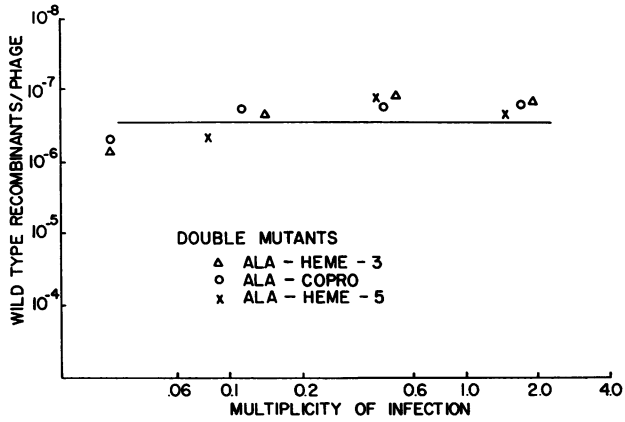

FIG. 2.—Frequency of recombination to wild type of double mutants with phage from wild-type donors at different multiplicities of infection.

TABLE 2. *Frequency of transduction to wild type with double mutants of S. aureus U-71.*

	Donor		
Recipients	ALA-copro	ALA-heme-2	ALA-heme-5
Copro-9	0*	455	290
Copro-40	0	460	200
Proto-44	120	140	90
Proto-50	180	190	135
Heme-2	240	0	7
Heme-3	260	0	5

* Transductants/10^8 phage scored in nutrient agar containing 8 μM ALA.

of recombination of the protoheme or copro lesion as tested by the ability to grow in the presence of ALA is about $190/10^9$ phage. This represents the frequency of a double crossover. A quadruple crossover would be expected to occur at a frequency of much less than 1 in 10^{13} and could not be detected. If the PBG locus were between the ALA and the copro or heme loci, a quadruple crossover would be necessary to recover wild-type recombinants. No wild-type recombinants were recovered in these experiments, although double crossovers occurred at the expected frequency. The heme locus appears to be on one side of the ALA-copro region as the recombinations to wild type occur at the frequency of double crossovers.

Genetic map of the heme locus: With linkage and gene order established, a genetic map was constructed using two factor crosses from the data of Table 1. The map distances expressed as recombinants to wild type per 10^8 phage are additive to within 10 per cent. In the map illustrated in Figure 3, genes specifying five enzymes in the heme biosynthetic pathway are tightly linked and are arranged in the sequence of metabolic reactions.

Small colony variants of *S. aureus* can be produced by many agents.[7] The protoheme-stimulated mutants selected in the presence of kanamycin are genetically stable and are neither dependent on nor resistant to kanamycin. The fact that revertants appear to be genetically like the wild type indicates that these mu-

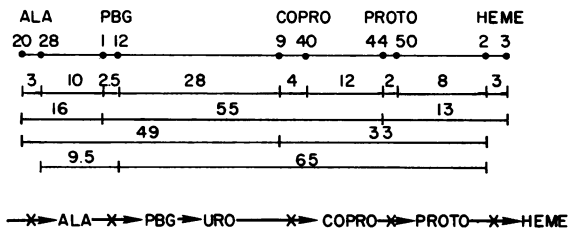

FIG. 3.—Genetic map of the heme locus of *S. aureus*.

TABLE 3. *Frequency of recombination between double mutants and phage from single mutants.*

	Donor phage	Recipient	Recombinants/10^9 Phage *A*	*B*
1.	ALA-20	ALA-copro	146*	0
2.	ALA-20	ALA-heme	280	0
3.	PBG-1	ALA-copro	146	0
4.	PBG-1	ALA-heme	184	0
5.	Copro-9	ALA-heme	151	0
6.	Heme-2	ALA-copro	161	140†
7.	Heme-2	ALA-heme	10	0

* Recombinants were scored in nutrient agar containing 8 μM ALA (col. *A*) and in nutrient agar (col. *B*). Assuming a gene order of ALA-PBG-copro-heme, column *A* represents the frequency of double crossovers, and column *B* represents the frequency of quadruple crossovers in experiments 3–5.

† In experiment 6, columns *A* and *B* represent the frequency of double crossovers.

tants are not episomal under the conditions of these experiments. Increasing the kanamycin concentration during the selection procedure increases the proportion of small-colony variants but reduces the number of survivors. Streptomycin and streptomycinlike drugs have been used to select mutants whose growth is stimulated by protoheme in *S. aureus*,[8] *Bacillus subtilis*,[9] and *Escherichia coli*.[10, 11] A mechanism by which streptomycinlike drugs should be effective in selecting protoheme mutants with such good efficiency is not readily apparent.

Unlike the genetic loci controlling protoheme biosynthesis in *S. aureus*, a genetic analysis of protoheme mutants of *E. coli* indicates that the ALA locus is not linked to three other genes involved in protoheme biosynthesis.[11]

The majority of the small-colony mutants selected by the kanamycin treatment are unable to form an electron transport system rapidly in the presence of protoheme. At least two classes of mutants whose growth is stimulated by protoheme represent different genes, as determined by the frequency of recombination to wild type. These two classes of mutants are not sufficiently closely linked to the heme loci to be cotransduced with the heme loci.

Summary.—A population of mutants of *S. aureus* unable to form an electron transport system rapidly can be selected by growth in the presence of kanamycin. About 20 per cent of the mutants form the electron transport system as rapidly as the wild type in the presence of protoheme. These protoheme mutants can be separated into five classes which represent five enzymatic lesions in the protoheme biosynthetic pathway. The genes specifying these enzymes are tightly linked and are cotransducible. These genes are arranged in the same sequence as the reactions in the biosynthetic pathway.

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