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Proposed Pathway of Triterpenoid Carotenoid Biosynthesis in *Staphylococcus aureus*: Evidence from a Study of Mutants

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Mutants of *Staphylococcus aureus* were isolated which showed changes in pigment composition compared with the parent strain. On the basis of differences in their triterpenoid carotenoid composition they were classified into seven types. In five of these types, there appeared to be a blockage in the biosynthetic pathway which resulted in the absence of some products and accumulation of others. The changes in the other two types appeared to be a consequence of some change in regulation. A scheme for the biosynthesis of triterpenoid carotenoids is presented in which the first C_{30} intermediate, 4,4′-diapophytoene, is converted via 4,4′-diapophytofluene, 4,4′-diapo-ζ-carotene, 4,4′-diaponeurosporene, 4,4′-diaponeurosporenal, 4,4′-diaponeurosporenoic acid, and glucosyl-diaponeurosporenoate to the major pigment staphyloxanthin.

We have previously described the isolation and identification of a series of carotenoids from *Staphylococcus aureus* (7) which are responsible for the color of pigmented cultures. In contrast to most naturally occurring carotenoids which possess a C_{40} skeleton, these carotenoids are triterpenoid, possessing a C_{30} skeleton. Taylor and Davies (R. F. Taylor and B. H. Davies, 4th Int. Symp. Carotenoids Abstr. Commun., p. 66-67, 1975) have already reported the isolation of some triterpenoid carotenoids from one strain of *S. aureus,* and Davies (3) has suggested a biosynthetic pathway for their formation analogous to the Porter-Lincoln pathway by which tetraterpenoid carotenoids are formed (8, 9). When grown under appropriate conditions, the majority of *S. aureus* strains are orange in color (2). From these strains, the major pigment which can be extracted is staphyloxanthin (J. H. Marshall and E. S. Rodwell, 3rd Int. Symp. Carotenoids Abstr. Commun., p. 56-57, 1972), which we have now characterized as a glucosyl diester of a triterpenoid carotenoid carboxylic acid and a fatty acid (7). Some naturally occurring strains differ in color, the differences reflecting qualitative or quantitative differences in carotenoid composition. Treatment of pigmented strains with mutagens may also produce organisms with altered pigment content (1, 5, 10). This paper reports the isolation and properties of several *S. aureus* mutants which differ from the parent strain in carotenoid composition. The results provide additional evidence for the pathway of biosynthesis of triterpenoid carotenoids in *S. aureus*.

MATERIALS AND METHODS

The organism used was *S. aureus* S41. Details about this organism, methods used for its growth and for extraction, and analysis of pigments have been described previously (7).

Mutants with different pigment patterns. Mutants were derived from the wild-type strain after exposure to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wis.). Cells from a culture in the late exponential phase of growth were collected by centrifugation, suspended in 0.04 M citrate (pH 5.0) containing nitrosoguanidine (100 μg/ml), and incubated with shaking at 37°C for 30 to 60 min. They were again collected by centrifugation and suspended in 0.04 M citrate, serial 10-fold dilutions were prepared, and 0.1-ml portions of appropriate dilutions were spread on glycerol monoacetate agar plates. After incubation at 37°C for 2 to 3 days, colonies which differed in color from wild-type colonies were selected for further study. The mutants were characterized initially by growing them in glycerol monoacetate broth (100 ml) for 24 h, extracting the pigments, and analyzing the extracts by thin-layer chromatography, using as the stationary phase silica gel and as the mobile phase light petroleum-acetone in the ratio 99:1 (vol/vol) for solvent i or 13:7 (vol/vol) for solvent iii (7).

RESULTS

Selection of mutants. By subjecting the wild-type strain to the mutagenic action of nitrosoguanidine and selecting colonies the color of which differed from that of the wild-type colonies, mutants were obtained which ranged in color from white through various intensities of yellow and orange to deep orange. An analysis of the carotenoid content of 455 such mutants

enabled us to group them into seven types (Table 1). Six of these types were obtained after treatment of the wild-type strain with the mutagen and selection of differently colored colonies. It is possible, however, that mutants might occur with altered pigment patterns which produce colonies indistinguishable in color from that of the wild type. To guard against the possibility that mutants of this type were not being detected, a mutant selection experiment was carried out in which a yellow-pigmented type V mutant was treated with the mutagen; this yielded a number of orange colonies, most of which proved upon analysis to belong to a seventh mutant type, type IV. Although the color of mutant colonies of this type is very similar to that of wild-type colonies, it is possible, after some practice, to distinguish between them, and one type IV mutant was subsequently isolated directly, after treatment of the wild type with the mutagen.

Properties of mutants. Within each mutant type the carotenoid pattern was the same, but the content varied considerably (Table 1). In type I, no carotenoids were found; in types II to V, one or more, but not all, of the carotenoids found in the wild type were present; in types VI and VII, the pattern was unchanged, but the total carotenoid content was either less (type VI) or greater (type VII) than that of the wild type.

A more detailed analysis of the carotenoid composition of a strain from each of mutant types I to V is shown in Table 2, where the corresponding figures for the wild-type strain are also given. Although types I and II produced white colonies, they differed in that type I contained no carotenoids, whereas type II contained considerably more 4,4'-diapophytoene than the wild type but no other carotenoid. Colonies of type III mutants are yellow, the major carotenoid produced being 4,4'-diaponeurosporene accompanied by minor quantities of other caro-

TABLE 2. *Quantitative carotenoid analysis of one strain each of mutant types I to V and of the wild-type strain*

Carotenoid	Carotenoid (μg/g [dry wt])					
	Wild type	Mutant type				
		I	II	III	IV	V
4,4'-Diapophytoene	40	—[a]	220	70	31	35
4,4'-Diapophytofluene	3	—	—	5	2	2
4,4'-Diapo-7,8,11,12-tetrahydrolycopene	2	—	—	5	1	1
4,4'-Diapo-ζ-carotene	6	—	—	18	4	4
4,4'-Diaponeurosporene	10	—	—	117	8	9
Neo-4,4'-diaponeurosporene B	1	—	—	11	1	1
Neo-4,4'-diaponeurosporene C	1	—	—	7	1	1
4,4'-Diapolycopene	—	—	—	2	—	—
Total carotenes	63	—	220	235	48	53
4,4'-Diaponeurosporenal	1	—	—	—	110	2
cis-4,4'-Diaponeurosporenal	—	—	—	—	25	—
4,4'-Diapolycopenal	—	—	—	—	3	—
4,4'-Diaponeurosporenoic acid	38	—	—	—	—	120
cis-4,4'-Diaponeurosporenoic acid	2	—	—	—	—	5
Staphyloxanthin	360	—	—	—	—	—
Isostaphyloxanthin	10	—	—	—	—	—
Glucosyl-diaponeurosporenoate	10	—	—	—	—	—
Hydroxy-400 compounds	10	—	—	35	10	10
Total xanthophylls	431	—	—	35	148	137
Total carotenoids	494	—	220	270	196	190
Carotenoid (nmol/g [dry wt])	745	—	540	670	465	450

[a] —, None detected.

TABLE 1. *Carotenoid composition of 455 S. aureus mutants with changed pigment patterns*

Organism	No. of mutants examined	Color of colonies	Principal carotenoids present	Total carotenoids (μg/g [dry wt])
S41 (parent strain)		Orange	Complete series[a]	494
Mutant type				
I	80	White	None	0
II	65	White	4,4'-Diapophytoene	10–220
III	150	Pale yellow	Carotenes[b]	15–270
IV	35	Orange	Carotenes[b] 4,4'-Diaponeurosporenal	50–196
V	55	Deep yellow	Carotenes[b] 4,4'-Diaponeurosporenoic acid	35–190
VI	60	Buff to pale orange	As S41	10–250
VII	10	Deep orange	As S41	620–1,150

[a] 4,4'-Diapophytoene, 4,4'-diapophytofluene, 4,4'-diapo-ζ-carotene, 4,4'-diaponeurosporene, 4,4'-diaponeurosporenoic acid, glucosyl-diaponeurosporenoate, staphyloxanthin, isostaphyloxanthin.

[b] 4,4'-Diapophytoene, 4,4'-diapophytofluene, 4,4'-diapo-ζ-carotene, 4,4'-diaponeurosporene.

tenes but no xanthophylls except small and variable amounts of the "hydroxy-400" group (7). The principal carotenoid of type IV mutants was the aldehyde 4,4'-diaponeurosporenal, present in barely detectable amounts in the wild type, whereas the principal carotenoid of type V mutants was 4,4'-diaponeurosporenoic acid.

The properties of mutant types I to V suggested that they each differed from the parent strain in having lost the ability to carry out one step in carotenoid biosynthesis as a consequence of loss of function of a structural gene, that this blockage resulted in accumulation of the substrate of the blocked reaction, and that each mutant type was blocked at a different step. Additional evidence of the sequence of these steps was obtained by isolating mutants with more than one mutational change affecting the pathway. From a type V mutant, double mutants were obtained with phenotype I, II, III, or IV; a type IV mutant yielded mutants with phenotype I, II, or III; a type III mutant yielded mutants with phenotype I or II; a type II mutant yielded only mutants with phenotype I. The changes which occurred in mutant types VI and VII did not appear to change the abundance of any specific intermediate of the pathway, but modified its overall regulation.

The conversion of 4,4'-diapophytoene to 4,4'-diaponeurosporene via the intermediates 4,4'-diapophytofluene and 4,4'-diapo-ζ-carotene (or its isomer 4,4'-diapo-7,8,11,12-tetrahydrolycopene) would involve three successive removals of 2H. If each step is a separate enzymatic reaction, it should be possible to isolate mutants blocked at each one of these steps. Of the 295 mutants listed in Table 1 which were white or pale yellow, the white ones produced no carotenoids (type I) or 4,4'-diapophytoene only (type II), whereas the pale-yellow ones produced predominantly 4,4'-diaponeurosporene, with smaller quantities of the intermediate carotenes (type III). In an attempt to obtain mutants blocked at one of the intermediate stages, we examined a further 150 white or pale-yellow mutants; all of these also fell into one of the same three types.

These mutants with modified carotenoid patterns provided convenient sources of some of the carotenoids for chemical investigation. Some type VII mutants produced two to three times as much total carotenoid as did the wild type. Other types accumulated specific compounds in much larger quantities than did the wild type (Table 2). This property was particularly useful in providing sufficient 4,4'-diaponeurosporenal from a type IV mutant for its isolation and identification before its occurrence, in trace amounts, in the wild type was detected (7).

The presence of squalene in *S. aureus* was reported by Suzue et al. (11). We found about 20 μg/g (dry weight) in both exponential-phase and stationary-phase cells of strain S41 (9) and about the same amount in type II mutants. In 50 type I mutants examined, all contained some squalene, the amount varying from 2 to 70 μg/g (dry weight).

The major carotenoid of type III mutants was 4,4'-diaponeurosporene, accompanied by some 4,4'-diapo-ζ-carotene, 4,4'-diapophytofluene, and 4,4'-diapophytoene. Small quantities of an additional carotene, found only in these mutants and not in the wild type or other mutants, was shown to be the dehydrogenation product of diaponeurosporene, 4,4'-diapolycopene (7). Some analyses also showed the presence of small but variable amounts of the hydroxy-400 xanthophylls. It was only type III mutants which produced significant amounts of these products, and sufficient material was obtained from them for limited chemical investigation (7). The major product found in all of these mutants was 4,4'-diaponeurosporene, and the fact that no mutants were found which produced the hydroxy-400 group of compounds as major products suggests they are not normal metabolic intermediates. In one type III mutant investigated in more detail, hydroxy-400 compounds comprised 2% of the total carotenoid in cells from an 18-h culture, increasing to 13% after 48 h, whereas the diaponeurosporene content remained at 50%. Several colorless fluorescent components were also found in trace amounts in this mutant, and enough of one of them was obtained from a 5-liter culture to obtain an absorption spectrum which showed maxima at 348, 375, and 398 nm, close to those of a phytofluenol-like derivative detected in *S. aureus* by Hammond and White (6). The hydroxy-400 compounds and the fluorescent compounds are probably degradation products of carotenes. We could obtain products similar to the hydroxy-400 compounds in about 5% yield by bubbling air through a solution of pure 4,4'-diaponeurosporene in methanol at 50°C for 15 min or by spotting 4,4'-diaponeurosporene on silica gel thin-layer plates and heating to 60°C before thin-layer chromatography.

Although most of the carotenoid found in type IV mutants was 4,4'-diaponeurosporenal, small amounts of the more unsaturated aldehyde 4,4'-diapolycopenal were also present, together with carotenes in quantities similar to those present in the parent strain. The major product of type V mutants was 4,4'-diaponeurosporenoic acid; it was accompanied by carotenes and 4,4'-diaponeurosporenal in quantities similar to those in the parent strain.

Mutant types sought for unsuccessfully.

Since 4,4'-diaponeurosporenol has been shown to be an intermediate produced in significant amounts by *Streptococcus faecium* (13), it was anticipated that it would also be found in *S. aureus* as an intermediate between 4,4'-diaponeurosporene and 4,4'-diaponeurosporenal. Attempts to detect it in the wild type were unsuccessful (7), and a search for it among a series of yellow mutants in which metabolic blocks might have led to its accumulation also failed.

It should also be possible to obtain mutants unable to convert glucosyl-diaponeurosporenoate to staphyloxanthin, but no selection on the basis of colony color would be possible since the absorption spectra of the two compounds are identical. A series of 50 orange colonies was selected after treatment of the wild-type strain with nitrosoguanidine; analysis of their carotenoid patterns showed that none was this type of mutant. Clearly, it will be necessary to screen a much larger number to have a reasonable chance of isolating such a mutant.

DISCUSSION

On the basis of the results described here, together with those reported in the accompanying paper (7), we can now confirm and extend the pathway proposed earlier (J. H. Marshall and G. J. Wilmoth, 5th Int. Symp. Carotenoids Abstr. Commun., p. 36, 1978) for the biosynthesis of the triterpenoid carotenoids of *S. aureus*. This pathway is shown in Fig. 1.

As discussed previously (3, 4, 7), the most probable route to the formation of the C_{30} chain is by condensation of two molecules of farnesyl pyrophosphate, a mechanism analogous to the formation of phytoene by condensation of two molecules of geranylgeranyl pyrophosphate. Although squalene occurs in both the wild-type and mutant strains and is known to be formed in other organisms by condensation of two molecules of farnesyl pyrophosphate, no mutants were obtained whose behavior was compatible with squalene being an intermediate in carotenoid biosynthesis. It seems probable that the first C_{30} intermediate produced is 4,4'-diapophytoene in the same way as in the C_{40} series, where the first intermediate is phytoene and not lycopersene (4).

The results suggest that the three successive dehydrogenation steps by which 4,4'-diapophytoene is converted to 4,4'-diaponeurosporene are probably catalyzed by a single dehydrogenase enzyme with relatively broad specificity. Type III mutants which possess this enzyme produce as their main product 4,4'-diaponeurosporene, with smaller amounts of 4,4'-diapo-ζ-carotene, 4,4'-diapo-7,8,11,12-tetrahydrolycopene, and 4,4'-diapophytofluene, whereas type II mutants which lack it produce only 4,4'-diapophytoene; no mutant blocked at any stage between these two types was found. From 4,4'-diapophytofluene to 4,4'-diaponeurosporene the main pathway goes through 4,4'-diapo-ζ-carotene, but the alternative path through the unsymmetrical 4,4'-diapo-7,8,11,12-tetrahydrolycopene also operates. Taylor and Davies (12) reported both pathways in *Streptococcus faecium* but could not detect the unsymmetrical heptaene in *S. aureus*; our results show that it is present in *S. aureus*, and the mechanisms of the dehydrogenation reactions are probably identical in the two organisms. The small amount of the fully unsaturated 4,4'-diapolycopene found in type III mutants is also probably produced by the action of the same dehydrogenase on diaponeurosporene, although its ability to attack the final 7,8- bond must be considerably less than its activity in the earlier steps, since only when the 4,4'-diaponeurosporene concentration is relatively high, i.e., when the enzyme responsible for its further metabolism is missing, is the further dehydrogenation product 4,4'-diapolycopene detectable.

The next stages in the metabolic pathway do not involve any further change in the carbon chain but involve introduction of oxygen functions probably through the action of mixed-function oxidases on the terminal methyl group to give the aldehyde and then the carboxylic acid. Mutant types IV and V are blocked at these steps. The pathway and end products in *S. aureus* thus differ from those found in *Streptococcus faecium* (13), in which the major xanthophylls are 4,4'-diaponeurosporenol and its glucoside. We were unable to detect any 4,4'-diaponeurosporenol in *S. aureus*, either in the parent organism or in any mutants, in spite of specific searches for it and attempts to isolate mutants accumulating it. If it is an intermediate in the conversion of 4,4'-diaponeurosporene to 4,4'-diaponeurosporenal, it must remain firmly enzyme bound and never be present in the free form in sufficient quantities to be detected.

The conversion of 4,4'-diaponeurosporenoic acid to staphyloxanthin requires esterification of two glucose hydroxyl groups with 4,4'-diaponeurosporenoic acid and with the fatty acid 12-methyltetradecanoic acid, reactions which would be expected to proceed via specific acyl coenzyme A derivatives. There is no evidence as to the nature of the enzymes involved, although the isolation of glucosyl-diaponeurosporenoate suggests that it is probably an intermediate.

The establishment of a biosynthetic pathway by isolation and characterization of appropriate mutants has been widely used. When the pathway involves metabolites essential for the growth of the organism so that mutants with

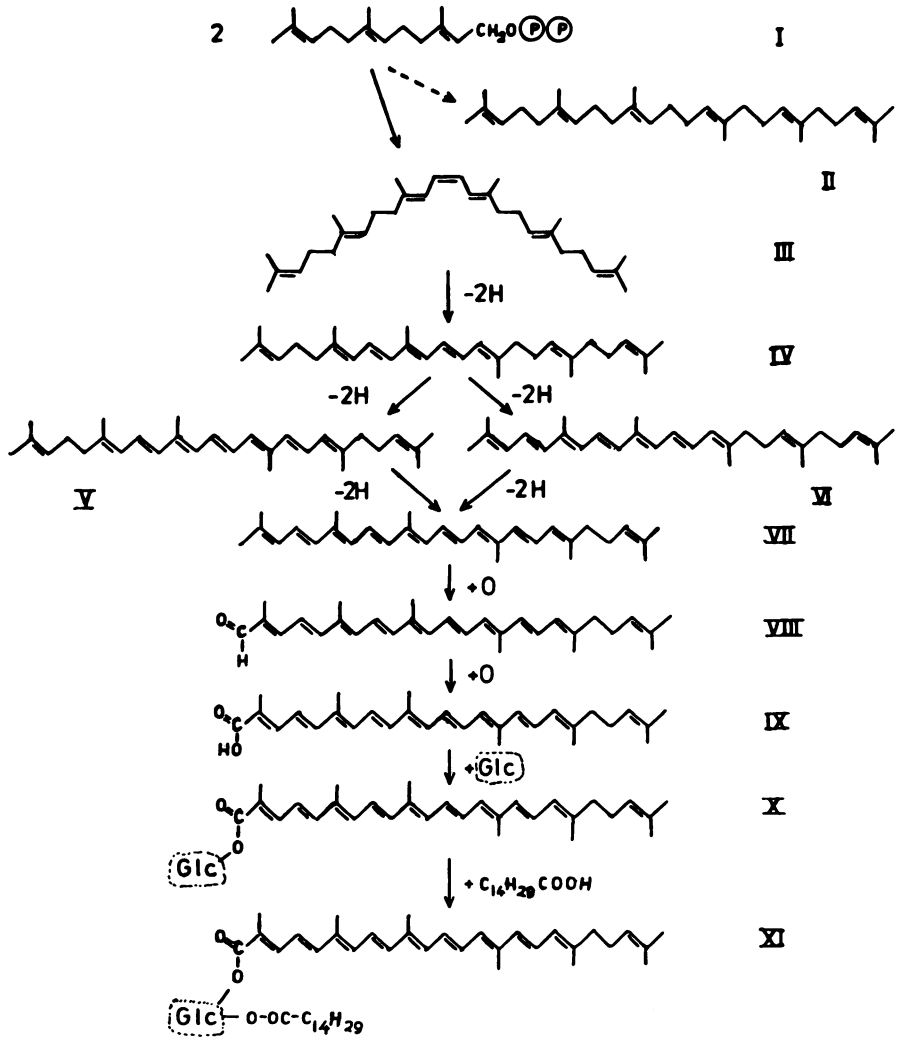

FIG. 1. *Proposed pathway for biosynthesis of triterpenoid carotenoids in S. aureus. (I) Farnesyl pyrophosphate; (II) squalene; (III) 15-cis-4,4'-diapophytoene; (IV) 4,4'-diapophytofluene; (V) 4,4'-diapo-ζ-carotene; (VI) 4,4'-diapo-7,8,11,12-tetrahydrolycopene; (VII) 4,4'-diaponeurosporene; (VIII) 4,4'-diaponeurosporen-4-al; (IX) 4,4'-diaponeurosporen-4-oic acid; (X) glucosyl-diaponeurosporenoate; (XI) staphyloxanthin.*

defects in the pathway are auxotrophic, this provides a convenient method of selection. For pathways involving secondary metabolites, however, no such selective method is available. For mutants with defects in carotenoid biosynthesis, we were able to make use of differences in colony color to select mutants blocked at different steps of the pathway. The main limitation of this method is the fact that not all changes in carotenoid composition lead to distinguishable differences in the color of colonies.

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