Classification of Vitamin K-deficient Mutants of *Staphylococcus aureus*

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SUMMARY

Twelve menaquinone-lacking mutants of *Staphylococcus aureus*, selected by neomycin, could be classified according to the point of their metabolic block. The mutants of class I were affected prior to the synthesis of shikimic acid, those of class II at a point following the synthesis of shikimic acid, and class III after the separation of the paths of aromatic amino acid biosynthesis but prior to the formation of the naphthoquinone ring. Mutants of class IV were probably affected at the level of synthesis of the isoprenoid side chain of menaquinone.

INTRODUCTION

The vitamin K-deficient mutants of *Staphylococcus aureus*, recently isolated by means of neomycin (Săsărman *et al.* 1968; Săsărman *et al.* 1969), afford an ideal means for studying menaquinone biosynthesis, since staphylococci, like the other Gram-positive cocci, possess only one type of quinone (Bishop, Pandya & King, 1962; Jeffries *et al.* 1967). The early stages of menaquinone biosynthesis are common to the biosynthesis of the aromatic amino acids (Cox & Gibson, 1964, 1966) up to the point of formation of chorismate or prephenate. Thereafter, the path diverges and the steps in the formation of the naphthoquinone ring are poorly understood (Gibson & Pittard, 1968; Zenk & Leistner, 1968). For identification of the unknown steps in menaquinone synthesis, only the mutants affected at some specific stages of the synthesis of the naphthoquinone nucleus are important. Our study was undertaken in order to identify such mutants among catalase-positive dwarf colony mutants of *S. aureus* and to try to classify the vitamin K-deficient mutants of *S. aureus* in several groups according to the point of their metabolic block.

METHODS

Strains. The menaquinone-negative (*meq*⁻) mutants investigated were derived from the following normal *Staphylococcus aureus* strains maintained in the collection of the Dr I. Cantacuzino Institute, Bucharest: *S. aureus* 3c, 11s, NINA, 1363. All these strains were isolated from hospital patients in Bucharest.

Isolation and characters of the meq⁻ mutants of *Staphylococcus aureus*

(a) *Selection of mutants.* The *meq*⁻ mutants of *Staphylococcus aureus* were obtained by selection with neomycin, as previously described (Sărsăman *et al.* 1968). On nutrient agar plates containing neomycin these mutants appeared as dwarf colonies similar to haemin-deficient mutants. Differentiation from the latter was carried out on the basis of catalase production.

(b) *Catalase production.* This was tested by placing a drop of H₂O₂ (3 %, v/v) on the surface of a 48 h. culture of the appropriate mutant growing on agar. The rapid appearance of gas bubbles indicated the presence of catalase activity in the mutant.

(c) *Stimulation by menadione.* To test the influence of menadione on growth and on vitamin K₂ synthesis, the mutants were grown on nutrient agar or on a synthetic medium supplemented with 1 µg. menadione/ml. The cultures were incubated at 37° for 48 h., together with controls grown in the absence of menadione.

(d) *Stimulation by shikimic acid.* To test the influence of this substance on growth and on vitamin K₂ synthesis, the mutants were grown on nutrient agar supplemented with 300 µg. shikimic acid/ml. The cultures were incubated at 37° for 48 h., together with a control cultured in the absence of shikimic acid.

(e) *Nutritional requirements of the mutants.* To compare the nutritional requirements of the mutant and parental strains, the synthetic medium described by Weaver & Pattee (1964) was supplemented with 5 µg. *p*-aminobenzoic acid + 1 µg. menadione/ml. On this medium (complete synthetic, or C medium) all the menadione-stimulated mutants grew normally with the exception of mutant SHSL 1, a derivative of *Staphylococcus aureus* strain 3c, which was also unable to develop on this medium. By excluding either menadione or tryptophan, it was possible to test to what extent the mutation had involved menaquinone synthesis only or the synthesis of all aromatic compounds. The original strains, with the above-mentioned exception, grew in the absence of both substances.

Extraction and determination of menaquinones

(a) *Chemicals.* The chemicals used were of the highest purity obtainable commercially and were not further purified. Standard K₂ (30) vitamin was a gift from Dr Isler, Hoffmann-LaRoche Ltd, Basle, Switzerland. The petroleum ether used throughout was b.p. 60 to 110°.

(b) *Spectroscopy.* The ultraviolet spectra were measured in a Unicam model SP 800 recording spectrophotometer.

(c) *Extraction of naphthoquinones.* Cultures grown for 48 h. on nutrient agar were suspended in normal saline and washed twice by centrifugation. Where indicated, the nutrient agar was supplemented with 1 µg. menadione/ml. or 300 µg. shikimic acid/ml. The wet cells (50 to 100 ml.) were successively extracted, first with two portions of caetone (150 ml.) and then with anhydrous ethyl ether (150 ml.). For each extraction 50 mg. of pyrogallol were added to the mixture, which was mechanically stirred while

being heated under reflux in a water bath for 90 min. The pooled extracts were concentrated under reduced pressure to approximately 50 ml. by heating in a water bath under a stream of nitrogen and then extracted with four portions of petroleum ether (100 ml.). This extract was concentrated under reduced pressure in a water bath under a stream of nitrogen and the residue dried to constant weight *in vacuo* over P_2O_5 .

(d) *Chromatography*. For chromatographic analysis the dried residue was dissolved in petroleum ether to a final concentration of 30 mg./ml. Analytical thin-layer chromatography was carried out on Chrom AR^r sheet 500 (Mallinckrodt Chemical Works Ltd, Montreal, Canada), using petroleum ether + chloroform (1:4, v/v) as the developing solvent, and the chromatograms were examined under u.v. light (253.7 nm.). Under these conditions K2 (30) gave a dark grey spot with R_F value of 0.9. Preparative thin-layer chromatography was carried out on Chrom AR^r sheet 1000 using the same solvent as above. The area of the chromatogram containing the menaquinones was removed and eluted with ethyl ether. The other solution was concentrated, re-chromatographed, and the adsorbent eluted as above. The ether extract was concentrated and the residue dried to constant weight *in vacuo* over P_2O_5 . Hexane solutions were used for u.v. analysis.

RESULTS AND DISCUSSION

Selection by means of neomycin and testing for catalase production resulted in the isolation of a large number of catalase-positive dwarf colony mutants of *Staphylococcus aureus*. With three exceptions, only those mutants that proved comparatively stable (during subsequent passages) were used in further experiments. A total of 12 catalase-positive colony mutants of *S. aureus* were included in the investigation; their origin and characteristics are shown in Table 1. Testing for the presence of vitamin K2,

Table 1. *Origin and characters of the catalase-positive dwarf colony mutants of Staphylococcus aureus*

Mutant	Parental strain no.	Presence of vitamin K 2	Stimulation by	
			Shikimic acid	Menadione
SHSL 1	3C	—	+	+
SHSL 2	11S	—	—	+
SHSL 3	NINA	—	—	+
SHSL 4	11S	—	—	+
SHSL 5	NINA	—	—	—
SHSL 6	1363	*	+	+
SHSL 7	11S	*	+	+
SHSL 8	1363 (Sm ^r)	*	—	+
SHSL 11	1363	—	—	+
SHSL 13	1363 (Sm ^r)	—	—	+
SHSL 14	NINA	—	—	—
SHSL 15	NINA	—	—	—
.	3C	+	.	.
.	11S	+	.	.
.	NINA	+	.	.
.	1363	+†	.	.

* Presence of vitamin K2 not tested because of frequent reversion of the mutants.

† The substance extracted has the same R_F as standard vitamin K2 (thin-layer chromatography) but a different spectrum.

in nine of these mutants revealed the absence of menaquinones (Table 1). In the other three mutants (SHSL 6, SHSL 7, SHSL 8) such tests could not be carried out because of frequent reversion.

Supplementation of the medium with shikimic acid revealed the existence of three mutants (SHSL 1, SHSL 6, SHSL 7) that were stimulated by this metabolite. Since this indicates a mutation involving some stage prior to the synthesis of shikimic acid, we concluded that the mutants SHSL 6 and SHSL 7, in which testing for vitamin K₂ could not be carried out, were also menaquinone-deficient mutants (*meq*⁻; cf. Sărsăman *et al.* 1968).

Fig. 1. Spectra of menaquinones isolated by preparative thin-layer chromatography from extracts of *Staphylococcus aureus*. —, Strain SHSL 1 grown on nutrient agar + menadione, 1 μ g./ml.; ---, strain 3c grown on nutrient agar;, vitamin K₂ (30) standard.

Examination of the influence of menadione on the growth of the *meq*⁻ mutants revealed that most, including the mutant SHSL 8 in which testing for vitamin K₂ was not feasible, were stimulated by this substance. Mutants grown in the presence of menadione form a compound with the R_F of vitamin K₂ (30) (Sărsăman *et al.* 1969; see also Hammond & White, 1969), and this suggests that menadione is an intermediate in menaquinone synthesis as reported by Zenk & Leistner (1968). However, examination of the substance synthesized by the mutants in the presence of menadione revealed a spectrum differing from that of vitamin K₂ (Fig. 1). In some instances this spectrum resembled that of a quinone extracted from the normal *Staphylococcus aureus* strain 1363, which also has the R_F value of standard vitamin K₂ (30) but a different spectrum. It seems likely therefore that menadione is an analogue of some intermediate in menaquinones biosynthesis, probably of the first compound of the pathway possessing a naphthoquinone ring. Consequently, in menadione-stimulated mutants the mutation involves a stage prior to the biosynthesis of the naphthoquinone ring, whereas in mutants that are not stimulated by menadione some later step, related to the synthesis of the isoprenoid side chain of menaquinones, is probably affected.

The nutritional requirements of *meq*⁻ mutants and parental strains were compared to differentiate, among the menadione-stimulated mutants, those affected before from those affected after the separation of the aromatic metabolic paths. A requirement for tryptophan in *meq*⁻ mutants indicated that the common part of the aromatic metabolic path had been affected. As was to be expected, the mutants stimulated by shikimic acid did not develop in the absence of tryptophan (Table 2). The mutant SHSL 8 was likewise unable to develop without tryptophan, so the lesion must also be within the common part of the metabolic path, at a stage subsequent to the synthesis of shikimic acid. The remaining mutants, which grew in the absence of tryptophan, were probably affected subsequent to the separation of the specific metabolic path in the synthesis of the naphthoquinone ring.

Table 2. *Nutrient requirements of meq*⁻ mutants of *Staphylococcus aureus*

Mutant	Parental strain no.	Growth on synthetic medium C*		
		Complete	Without menadione	Without tryptophan
SHSL 1	3c	-†	-	-
SHSL 2	11s	+	-	+
SHSL 3	NINA	+	-	+
SHSL 4	11s	+	-	+
SHSL 6	1363	+	-	-
SHSL 7	11s	+	-	-
SHSL 8	1363 (Sm ^r)	+	-	-
SHSL 11	1363	+	-	+
SHSL 13	1363 (Sm ^r)	+	-	+
.	3c	-	-	-
.	11s	+	+	+
.	NINA	+	+	+
.	1363	+	+	+

* Medium C includes menadione and tryptophan.

† *Staphylococcus aureus* parental strain 3c does not grow on this medium.

Table 3. *Classification of the meq*⁻ mutants of *Staphylococcus aureus*

Class	Mutant	Stimulation by menadione	Ability to synthesize tryptophan	Stimulation by shikimic acid
I	SHSL 1	+	N.T.	+
	SHSL 6	+	-	+
	SHSL 7	+	-	+
II	SHSL 8	+	-	-
III	SHSL 2	+	+	-
	SHSL 3	+	+	-
	SHSL 4	+	+	-
	SHSL 11	+	+	-
	SHSL 13	+	+	-
IV	SHSL 5	-	.	-
	SHSL 14	-	.	-
	SHSL 15	-	.	-

N.T. = not tested (see text).

On the basis of the investigation reported here, the vitamin K-deficient mutants of *Staphylococcus aureus* selected by means of neomycin could be classified in four classes (Table 3). Only the mutants of the third and fourth classes are menaquinone-deficient in this strict sense of the term, the others being aromatic mutants affected at the level of the common metabolic path. The distribution of *meq*⁻ mutants (Table 3) shows that the third class predominate; these mutants are of particular value for the elucidation of the biosynthesis of the naphthoquinone ring.

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