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Pantothenate-requiring dwarf colony variants of *Staphylococcus aureus* as the etiological agent in bovine mastitis

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INTRODUCTION

A number of authors have described dwarf colony variants of *Staphylococcus aureus* (D forms) obtained from normal strains kept under adverse conditions, and in a few cases such variants have been isolated from infectious lesions; in the latter cases the cultures developed normal colonies when incubated under increased CO_2 pressure. The metabolic defect causing the unusual growth feature had not been identified in any case (for references see Sompolinsky, Ernst-Geller & Segal, 1967).

As far as we are aware, D forms of *S. aureus* as the etiological agent in bovine mastitis have hitherto only been described in Israel. Since our earlier communication (Lernau & Sompolinsky, 1962) mastitis due to D staphylococci has been observed in nearly 30 herds under intensive mastitis control programme, which is more than 35 % of the herds under this programme, and also in a few other herds. D staphylococci have not been isolated from ovine mastitis (Dr R. Tamarin, personal communication) nor have they been observed among more than 150,000 strains of human origin examined by the Staphylococcus Reference Laboratory of Israel.

Most of the D strains isolated were thiamineless, i.e. when thiamine HCl was added to sterile nutrient media the bacteria grew as normal colonies. Two distinct metabolic disorders have been observed in the thiamineless strains: (1) inability to concentrate the thiazole moiety of thiamine; and (2) inability to phosphorylate thiamine-pyrimidine (Sompolinsky *et al.* 1967). In two herds D strains with other metabolic disorders have been isolated. In this paper we shall describe a group of strains isolated from one herd, and discuss a few epidemiological observations of the infection.

MATERIALS AND METHODS

Milk samples were plated on Difco blood agar base enriched with 4·0 % washed sheep erythrocytes. *Streptococcus agalactiae* was used to potentiate the staphylococcal β-haemolysin in the warm phase incubation (camp reaction).

The isolated staphylococcal strains were cultivated on Difco nutrient agar, Difco tryptose phosphate agar and sheep blood agar; the last medium was composed

of beef broth essentially as described (Sompolinsky, Saruf & Glazewski, 1966) enriched with 4% washed sheep erythrocytes and solidified with Bacto agar.

The basic medium for examinations of vitamin requirements was prepared on the basis of vitamin-free casamino acids (Difco), purified with active carbon: Bacto vitamin-free casamino acids (purified), 25 g.; NaCl, 8 g.; KH_2PO_4, 1·2 g.; K_2HPO_4, 1·2 g.; Special Noble agar, Difco, 15 g.; distilled water to 1 l.; pH 7·2.

To this medium was added sterile solutions of thiamine HCl, nicotinic acid, biotine and L-cysteine to final concentrations of 10^{-6} (w/v) each. This medium will be designated CVF. Other supplements will be specified under Results. Most growth factors were purchased from Sigma Chemical Co. Pantoic acid was prepared by alkaline hydrolysis of DL-pantoyl lactone as indicated by Grula & Grula (1962).

RESULTS

Epidemiological observations

When dwarf staphylococci were first isolated, the herd consisted of 55 lactating cows, 28 of which were infected with *S. aureus* in at least one quarter of the udder. The udders from which D strains were isolated showed upon palpation a marked degree of parenchymal fibrosis, indicating a rather chronic state of infection. Before the first isolation of the D staphylococci, the infected cows were treated on several occasions by the intra-mammary route with penicillin G, streptomycin, neomycin and tetracycline. The staphylococcal variants to be described were isolated from four cows in January and February 1967, and at each subsequent examination of milk samples from the infected udder quarters until the cows were sold, in one cow (Geveret) during more than one year. When the mastitis milks were plated a pure culture of D colonies was obtained from some samples; others showed a few normal colonies among the dwarfs (Plate 1, Fig. 1).

In our experience, treatment with an antibiotic shown to be suitable by *in vitro* tests is successful in curing glands chronically infected with normal *S. aureus* in about 45% of cases. However, none of the glands infected with D-staphylococci were cured by similar treatment; this is in accordance with our experience with dwarf-staphylococcal mastitis in other herds.

Bacteriological examinations

Eight strains of *S. aureus*, isolated from four cows between January 1967 and February 1968 were included in this study (Table 1). These strains, with the exception of No. 164, grew as pin-point, transparent streptococcus-like colonies on nutrient agar, tryptose phosphate agar and sheep blood agar as well as many other nutrient media in general use. No. 164 produced colonies of almost normal size on these media (Plate 1, Fig. 2) but in spite of this, it was included in this study, since no growth was obtained on unsupplemented CVF and normal colonies developed on CVF + Ca-pantothenate (Table 1). No. 287 was also distinct from the other strains in that it grew with D colonies both on unsupplemented and on pantothenate-CVF. The colonies of No. 207 on sheep blood agar were smaller than those of the other strains and barely visible after 48 hr.

The microscopic morphology was characteristic for staphylococci in all the strains; they produced a wide zone of hot-cold haemolysis on sheep blood agar; the coagulase test was positive; they were negative for Tween-splitting; they were all susceptible only to phage 42E of the international set of typing phages; and all were susceptible to sulphathiazole, penicillin G, streptomycin, tetracycline,

Table 1. *Characterization of eight staphylococcal strains from bovine mastitis in one herd*

	Strains							
				Geveret				
Nutrient medium	Kafrit 164	Timna 207	Mafliga 266	195 RR*	287 RR	348 LR*	806 RR	1131 RR
Tryptose phosphate agar	N†	D†	D	D	D	D	D	D
Sheep blood agar	N	D	D	D	D	D	D	D
Vitamin-free Casamino agar (CVF)	Neg†	Neg	Neg	Neg	D	Neg	Neg	Neg
CVF + Ca-pantothenate	N	n†	N	N	D	N	N	N

* RR = right rear gland; LR = left rear gland of the udder.

† N = normal colonies. D = dwarf colonies. Neg = no growth.
n = colonies noticeably smaller than those of other pantothenateless strains on medium with equal concentrations of the vitamin.

Table 2. *Growth of D staphylococci on Ca-pantothenate and related compounds*

		Strains							
					Geveret				
Growth factor	Concentration*	Kafrit 164	Timna 207	Mafliga 266	195	287	348	806	1131
D-Ca-pantothenate	10^{-7}	++	+	++	++	D+	++	++	++
	10^{-9}	+	−	+	+	D	N.E.	+	+
β-alanine	10^{-4}	−	−	−	−	D	−	−	−
Pantoic acid	10^{-5}	+	+	+	+	D	+	+	+
DL-pantoyl lactone	10^{-4}	+	D	+	+	D	+	+	+
α-keto-iso-valeric acid	10^{-4}	−	−	−	−	D	−	−	−
D-pantothenyl alcohol	10^{-4}	D	−	+	−	D	−	−	−
D-pantethine	10^{-4}	++	+	++	++	D	++	++	++
	10^{-6}	+	−	+	+	D	+	+	+

* in w/v of the vitamin-free casamino agar medium.

++, growth of normal colonies in 24 hr.; +, growth of normal or sub-normal colonies in 72 hr.; D, growth of dwarf colonies; −, no growth; N.E., not examined.

chloramphenicol and erythromycin according to a diffusion test with sensitivity tablets (Rosco, Denmark) (Sompolinsky & Minkowski, 1969). After 48–72 hr. incubation on sheep blood agar, normal-sized colonies developed as papillae on some of the D-colonies. From these papillae, prototrophic, coagulase positive strains were isolated, which were susceptible to the same chemotherapeutic agents and typing phages as the progenitor strains.

None of the strains responded to the following supplements to CVF: Thiamine

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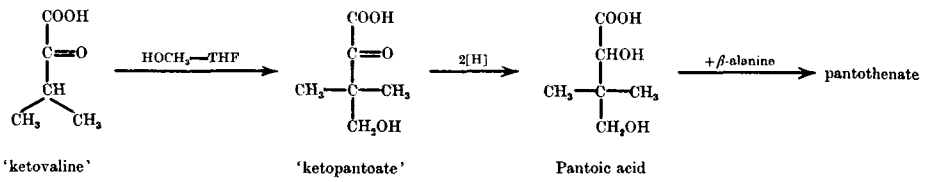
monophosphate (highest concentration tested 10^{-4}, w/v); thiamine pyrophosphate (10^{-4}); vitamin B_{12} (10^{-8}); nicotinamide (10^{-6}); vitamin K_1 (10^{-8}); ascorbic acid (10^{-7}); pyridoxal-HCl (10^{-5}); pyridoxamine di-HCl (10^{-7}); *p*-amino benzoic acid (10^{-7}); thioctic acid (10^{-4}); *i*-inositol (10^{-6}); and DL-threonine (10^{-4}). On CVF supplemented with Ca-pantothenate all strains, except No. 287, developed normal sized staphylococcal colonies in 24 hr (37°). In Table 2, the limiting concentration of pantothenate and response to related compounds is reported. As shown in the Table, the pantothenate-less strains responded also to pantethine (bis-(N-pantothenyl-amidoethyl) disulfide), DL-pantoyl lactone and pantoic acid (α-γ-hydroxy-β,β-dimethyl-butyric acid), prepared by alkaline hydrolysis of the pantoyl lactone. The concentration of pantoyl lactone required for delayed growth was a thousand times, and of pantoic acid a hundred times that of the concentration of Ca-pantothenate giving prompt growth (Plate 2). This may be due to differences in the speed of penetration through the cell membrane, which may also be responsible for the high concentration of pantethine required.

DISCUSSION

Wild strains of *Staphylococcus aureus* generally require the following vitamins for growth: thiamine or its thiazole + pyrimidine moieties, nicotinamide or nicotinic acid (Knight, 1937), and, for some strains, biotine. Requirement for Ca-pantothenate has hitherto not been observed in strains obtained from disease processes.

The biosynthesis of pantothenate in bacteria may be outlined as follows (Davis *et al.* 1968):



The pantothenate is subsequently condensed with cysteine to pantothenyl-cysteine from which pantethine is formed by release of CO_2.

The pantothenateless staphylococcal strains under consideration must be unable to add hydroxymethyl to 'ketovaline' or to reduce 'ketopantoate'. Since this latter compound was not available, we were unable to demonstrate which of these functions was affected.

The frequent occurrence of auxotrophic staphylococci in connexion with bovine mastitis in Israel is not easily explained. The possibility that a single auxotrophic bacterial cell arose by chance, multiplied in an udder gland and was then spread to other cows in the herd by the usual vehicles of infection, is unlikely in the case reported, since the pantothenate-requiring strains from different cows were not similar. No. 164 was distinguished from the other strains by its ability to develop almost normal colonies on tryptose phosphate and on sheep blood agar; any

unknown compound in these media might be used by this strain for production of pantothenate. Strain No. 266 from another cow seemed also to be specific in its ability to grow on pantothenyl alcohol, probably oxidizing it to pantothenate. No. 287, though isolated from the same udder gland as pantothenate-auxotrophs, required another compound that we so far have been unable to specify.

An alternative possibility for the simultaneous occurrence of a number of infections with D staphylococci in mastitis herds might be a selective advantage for the cocci with a specific metabolic disorder. This might be an *in vivo* analogy of 'penicillin-screening'. The fact that antibiotic treatment has always proved unsuccessful in infections with this kind of staphylococci might sustain this hypothesis, but we have so far no real experimental data supporting it further. In every case, some local factor, possibly an unknown mutagenic agent in the milk, must be an additional precondition for the development of these udder infections since they have not been described in other countries, though treatment of mastitis is similar in most areas of intensive milk production.

SUMMARY

In a herd affected with bovine mastitis, dwarf colony variants of *Staphylococcus aureus*, auxotrophic for Ca-pantothenate, were isolated from the milk of four cows with mastitis. From one of these cows, a dwarf variant with an unknown metabolic disorder was isolated during the same period. Infections with auxotrophic staphylococci were always chronic and proved to be refractory to antibiotic treatment, though the causative micro-organism was highly susceptible to the drugs used as judged by *in vitro* tests.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Plating of mastitis milk sample yielding D variant colonies of *Staphylococcus aureus* interspaced with a few normally sized colonies.

Fig. 2. Two pantothenate-less strains of *Staphylococcus aureus* on sheep blood agar (No. 1131 with pin-point colonies and No. 164 with almost normal colonies).

PLATE 2

Subcultures of the dwarf *Staphylococcus aureus*, strain Geveret 195, on: A, plain casamino acid medium (CVF); B, CVF + DL-pantoyl lactone (10^{-5}, w/v); C, CVF + pantoic acid (same conc.); and D, CVF + Ca-pantothenate (10^{-8}). Incubation: 48 hr. at 37° C.

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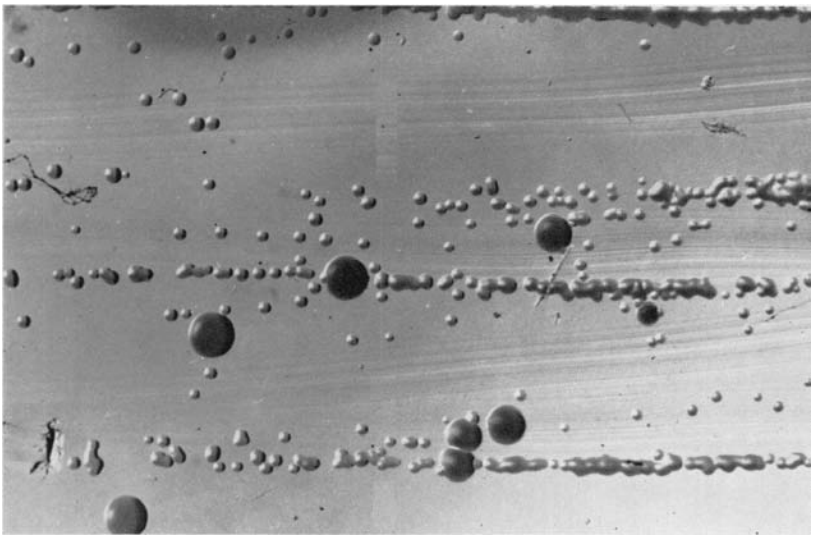

Fig. 1

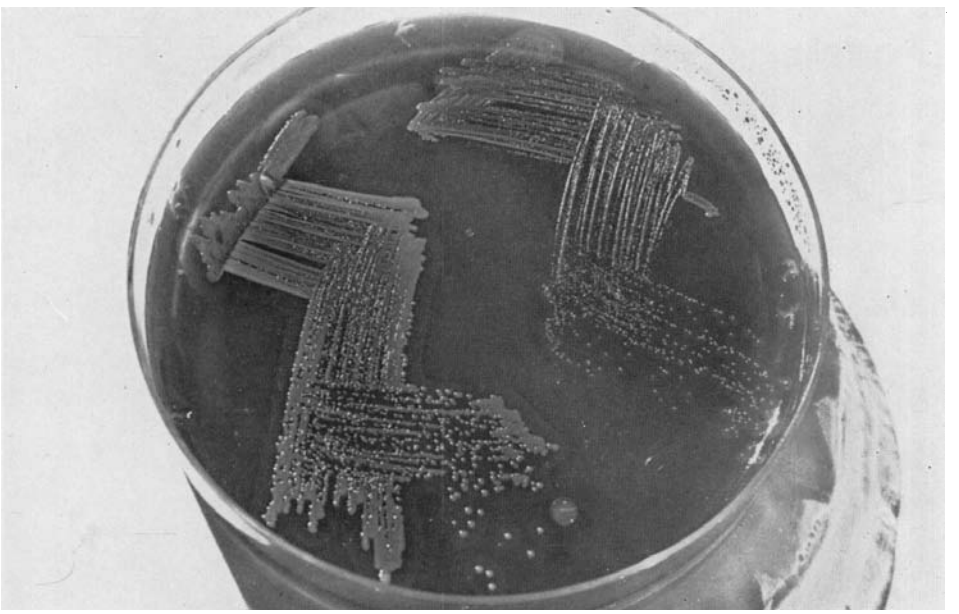

Fig. 2

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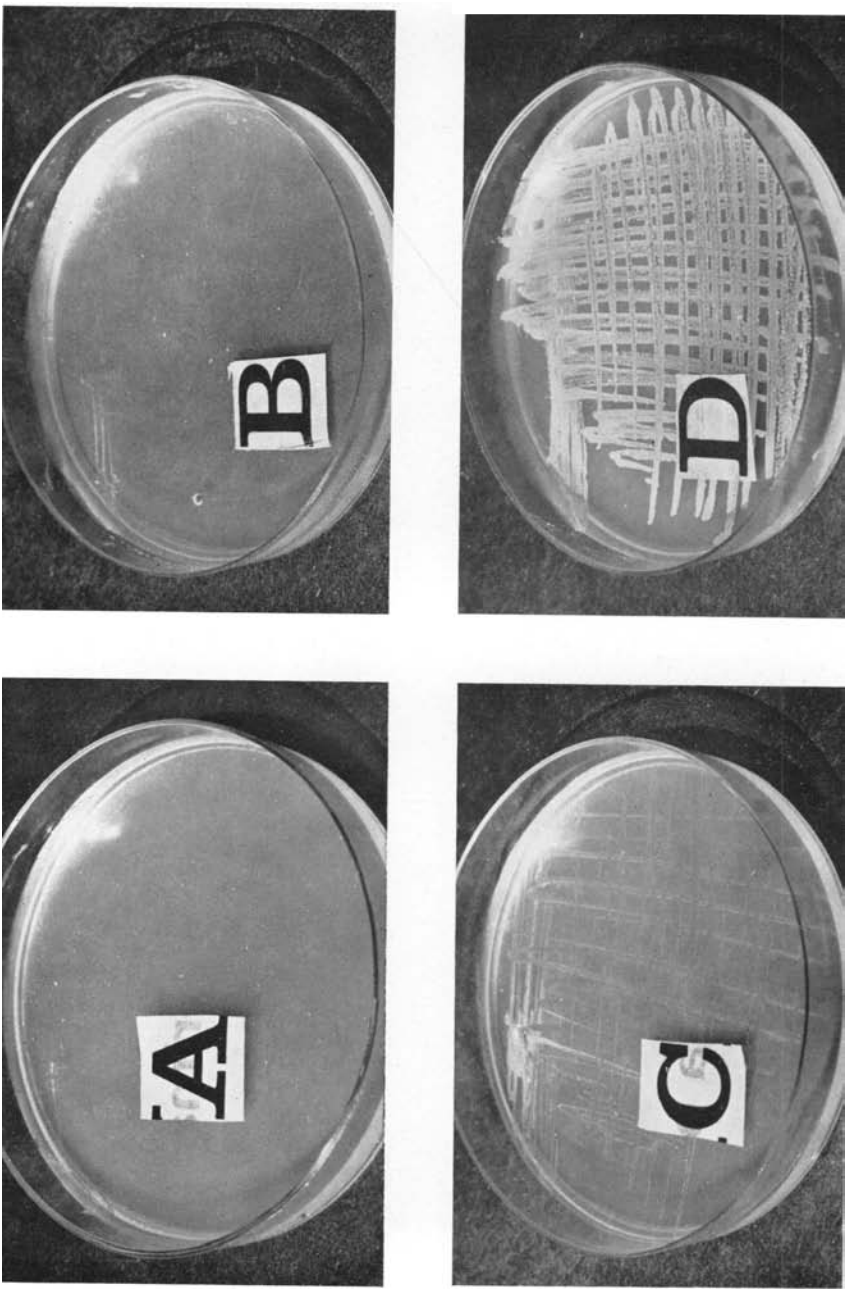

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