Additional Differentiating Characters of the Two Subspecies of *Staphylococcus hyicus*

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Forty-five strains of *Staphylococcus hyicus* subsp. *hyicus* and 36 strains of *S. hyicus* subsp. *chromogenes* were examined for bacteriolytic activity with the same assay system previously used in taxonomic studies on staphylococci. The two subspecies differed from each other chiefly in that for optimal lytic activity *S. hyicus* subsp. *hyicus* strains required a higher salt concentration in the test medium than *S. hyicus* subsp. *chromogenes* strains. The lack of lytic activity on B15TP1 medium was a major difference between *S. hyicus* and *S. aureus*, and the lack of activity on TP2P medium was a major difference between *S. hyicus* and *S. intermedius*. Penicillin-binding proteins (PBPs) were studied in 40 *S. hyicus* strains. The *S. hyicus* subsp. *hyicus* strains had only one PBP (mol. wt 79 000) while the *S. hyicus* subsp. *chromogenes* strains had three distinct PBPs (mol. wts 84 000, 82 000 and 79 000).

INTRODUCTION

Staphylococcus hyicus can be isolated from animals and includes coagulase-positive and coagulase-negative strains. Its role as the causative agent of porcine exudative epidermitis was described by Sompolinsky (1953). Further investigations confirmed its aetiological role in this disease (Jones, 1956; Mebus *et al.*, 1968; Hunter *et al.*, 1970), and implicated it in other infections of pigs (Phillips *et al.*, 1980) and cows (Brown *et al.*, 1967; Devriese & Derycke, 1979). Proposals for improving or simplifying the isolation and identification of these staphylococci in the veterinary diagnostic laboratory have been made (Devriese, 1977; Phillips & Kloos, 1981; Maddux & Koehne, 1982).

Taxonomically, *S. hyicus* has had a complicated history. It was originally described as *Micrococcus hyicus* (Sompolinsky, 1953), but in 1965 it was transferred to the genus *Staphylococcus* as *Staphylococcus* subgroup III (Baird-Parker, 1965). Subsequently, it was reclassified as *S. epidermidis* biotype 2 in the eighth edition of *Bergey's Manual of Determinative Bacteriology* (Baird-Parker, 1974). Although already in general use in veterinary publications, the name *S. hyicus* was formally proposed by Devriese *et al.* (1978), who also divided this strain into two subspecies, *S. hyicus* subsp. *hyicus* and *S. hyicus* subsp. *chromogenes*. The separation of the two subspecies was made on the basis of a number of phenotypic characteristics and DNA homology studies. Moreover, the known pathogenicity of *S. hyicus* can largely be ascribed to *S. hyicus* subsp. *hyicus*, whereas the pathogenic relevance of *S. hyicus* subsp. *chromogenes* is not yet clear.

Bacteriolytic (BA) activity has previously been used as a criterion in the classification of staphylococci of both human (Varaldo & Satta, 1978; Varaldo *et al.*, 1978*a, b*) and animal (Varaldo *et al.* 1978*b*) origin. The pattern of penicillin-binding proteins (PBPs), which are bacterial plasma membrane proteins that specifically and covalently bind β -lactam antibiotics,

Abbreviations: BA, bacteriolytic activity; PBP, penicillin-binding protein.

has been suggested to be a useful marker in staphylococcal taxonomy, and in general bacterial taxonomy (Fontana *et al.*, 1981; Tomasz, 1982; Waxman & Strominger, 1983). The aim of this study was to investigate a number of strains of the two *S. hyicus* subspecies for BA and PBP patterns.

METHODS

Bacterial strains. Eighty-one staphylococcal isolates were investigated. Forty-five *S. hyicus* subsp. *hyicus* strains were isolated from swine: five strains were from Czechoslovakia, twenty-five from Belgium, two (A2869c, S3588) from W. Germany, two (Hunter A and B) from England, two (ZH1029, ZH1037) from Switzerland, and nine from Yugoslavia. Thirty-six *S. hyicus* subsp. *chromogenes* strains, eighteen each from cattle and swine, were isolated in Belgium. The physiological properties of the strains had previously been investigated by Devriese *et al.* (1978).

Examination of BA. The BA patterns of the strains were determined with the assay system, composed of eight test media, which was used in previous taxonomic studies on staphylococci (Varaldo & Satta, 1978; Varaldo *et al.*, 1978*a, b*). The BA substrates, the test media, and the BA estimation were as described previously (Varaldo *et al.*, 1978*a*; Satta *et al.*, 1980).

Detection of PBPs. The strains to be examined for PBPs were first checked for penicillin-sensitivity using a standard agar diffusion procedure and commercial discs containing 10 units of penicillin G.

The bacteria were grown in brain heart infusion broth (Difco) at 37 °C on a gyratory water bath shaker (New Brunswick). In late exponential phase the cultures were quickly chilled, and the bacteria were harvested by centrifugation and washed twice with cold (4 °C) 0.01 M-sodium phosphate buffer (pH 7.2). The cells were then resuspended in 10 ml of the same buffer containing 150 µg lysostaphin ml⁻¹ (Sigma). After 20 min incubation at 37 °C, unlysed cells were removed by centrifugation at 5000 g for 10 min at 4 °C. Membranes were pelleted from the supernatants by centrifugation at 100 000 g for 30 min at 4 °C, washed twice with the phosphate buffer, and finally resuspended in the buffer at a protein concentration of 20 mg ml⁻¹. If not used immediately for penicillin binding assays, the membranes were stored at -70 °C until required.

Samples (50 µl) of membrane preparations were incubated with 10 µl [¹⁴C]penicillin G [50 µCi ml⁻¹, specific activity 53 mCi mmol⁻¹ (1.96 GBq mmol⁻¹; Amersham] at 37 °C for 15 min. The reaction was terminated by the addition of 5 µl unlabelled penicillin G (100 mg ml⁻¹). Then 25 µl 0.2 M-Tris buffer (pH 6.8) containing 6% (w/v) SDS and 6% (v/v) 2-mercaptoethanol were added to the samples, which were then boiled for 5 min in order to solubilize membranes (Satta *et al.*, 1980; Fontana *et al.*, 1983). Electrophoresis was done in 7% (w/v) polyacrylamide slab gels containing SDS according to Laemmli (1970). Reagents for SDS-PAGE, including SDS, were from Bio-Rad. For fluorography the gels were soaked in EN³HANCE (New England Nuclear) for 1 h and then washed in water for 1 h. Gels were dried under vacuum and then exposed to X-ray film (Kodak SO 282) for 30 d at -70 °C. PBPs were visualized in the developed films.

Molecular weights of PBPs were determined by co-electrophoresis with protein standards of known molecular weight.

RESULTS

Bacteriolytic activity

The BA of the 81 *S. hyicus* isolates was examined in the eight test media of the assay system. Seven BA patterns were found, four for *S. hyicus* subsp. *hyicus* and three for *S. hyicus* subsp. *chromogenes*. Of the 45 *S. hyicus* subsp. *hyicus* strains, 33 had the predominant BA pattern (no. 1), and of the 36 *S. hyicus* subsp. *chromogenes* strains, 26 had BA pattern no. 5 (Table 1).

Although the BA patterns of the strains of *S. hyicus* subsp. *hyicus* and of those of *S. hyicus* subsp. *chromogenes* were quantitatively different from each other, a certain qualitative similarity was evident. The strains of *S. hyicus* subsp. *hyicus* usually showed larger zones of transparency on T1 than on T0 medium, whereas the strains of *S. hyicus* subsp. *chromogenes* showed the opposite behaviour. This observation, as well as minor differences between the two subspecies usually observed on T3 and TP2 test media, may reflect the requirement for a higher salt concentration in the medium by *S. hyicus* subsp. *hyicus* for optimal lytic activity.

The BA patterns of the *S. hyicus* subspecies are shown in Table 2 and compared with those of the two coagulase-positive *Staphylococcus* species. Some similarities among these BA patterns are obvious. However, the lack of activity on B15TP1 medium was a major difference between *S. hyicus* and *S. aureus*, and the lack of activity on medium TP2P was a major difference between *S. hyicus* and *S. intermedius*.

Table 1. BA patterns of 45 strains of *S. hyicus* subsp. *hyicus* and 36 strains of *S. hyicus* subsp. *chromogenes*

Subspecies	Pattern no.	No. of strains	BA on the test media:							
			TP1	TP2	TP2P	T0	T1	T3	B15TP1	a61TP2
<i>S. hyicus</i> subsp. <i>hyicus</i>	1	33	++	+	—	+	++	±	—	—
	2	7	+	±	—	±	+	—	—	—
	3	3	++	+	—	++	++	±	—	—
	4	2	++	+	—	+	++	—	—	—
<i>S. hyicus</i> subsp. <i>chromogenes</i>	5	26	++	±	—	++	+	—	—	—
	6	8	++	±	—	+	+	—	—	—
	7	2	+	±	—	+	±	—	—	—

Table 2. BA patterns of *S. hyicus* and other coagulase-positive species

Species or subspecies	BA on the test media:							
	TP1	TP2	TP2P	T0	T1	T3	B15TP1	a61TP2
<i>S. hyicus</i> subsp. <i>hyicus</i>	++	+	—	+	++	±	—	—
<i>S. hyicus</i> subsp. <i>chromogenes</i>	++	±	—	++	+	—	—	—
<i>S. aureus</i> *	+++	+	—	±	++	—	+	—
<i>S. intermedius</i> *	++	+	+	+	++	—	—	—

* According to Varaldo *et al.* (1978*b*, 1980).

Penicillin-binding proteins

PBPs were investigated in 20 strains of *S. hyicus* subsp. *hyicus* and 20 strains of *S. hyicus* subsp. *chromogenes*. All strains tested displayed zones of inhibition ≥ 35 mm in diameter in disc susceptibility tests for penicillin G.

The PBP patterns were largely uniform within each subspecies. The strains of *S. hyicus* subsp. *hyicus* had a single PBP with an estimated molecular weight of 79 000. By contrast, the strains of *S. hyicus* subsp. *chromogenes* shared a PBP pattern characterized by three distinct bands with molecular weights of 84 000, 82 000 and 79 000. Thus, the single PBP of *S. hyicus* subsp. *hyicus* seemed to correspond to the lightest PBP of *S. hyicus* subsp. *chromogenes*. The different PBP patterns in strains of the two subspecies are shown in Fig. 1.

DISCUSSION

The BA patterns of *S. hyicus* subsp. *hyicus* and *S. hyicus* subsp. *chromogenes* are distinctly different from those observed in other staphylococci (Varaldo *et al.*, 1978*a, b*; 1980). The differences observed between the BA patterns of the two *S. hyicus* subspecies, although not marked, are nevertheless well defined in their dependence upon a different optimum salt concentration. It is noteworthy that in previous studies on staphylococcal lytic activity – dealing with both the zones of transparency displayed on solid test media (Satta *et al.*, 1977; Varaldo *et al.*, 1978*a*), and the characterization of purified lytic enzymes from different species (Valisena *et al.*, 1982) – the salt concentration proved to be one of the most significant factors affecting BA and helped to discriminate among different, yet related, staphylococcal lytic enzymes.

The different PBP patterns of the two *S. hyicus* subspecies are interesting in many respects. In *S. aureus*, the only staphylococcal species extensively investigated for PBPs, four distinct proteins have been identified, three with high molecular weights and one with a low molecular weight (Kozarich & Strominger, 1978; Georgopapadakou & Liu, 1980). No low-molecular-weight PBP was found in any of the *S. hyicus* strains examined. The occurrence of only one PBP in *S. hyicus* subsp. *hyicus* and three PBPs in *S. hyicus* subsp. *chromogenes* is particularly

Fig. 1. PBP patterns of nine strains of *S. hyicus* subsp. *hyicus* (A through I) and ten strains of *S. hyicus* subsp. *chromogenes* (J through S). The strains were: (A) SH66, (B) SH62, (C) SH59, (D) SH50, (E) SH1, (F) SH5, (G) SH16, (H) SH27, (I) SH150, (J) SH226, (K) SH225, (L) SH224, (M) SH223, (N) SH221, (O) SH210, (P) SH211, (Q) SH214, (R) SH219, (S) SH220.

significant taxonomically, showing both a difference between the subspecies and pattern uniformity within each subspecies. Taxonomic or phylogenetic relationships might be suggested by the fact that both subspecies share a PBP of molecular weight 79000. However, a wider investigation in progress in our laboratories shows that in staphylococci, PBPs are most often concentrated within a relatively narrow size range (molecular weight about 80000). Preliminary results suggest that the total number of PBPs in staphylococci is unusually low compared with the number in most other bacteria so far examined (Fontana *et al.*, 1981).

The present results contribute to a more consistent biological and taxonomic characterization of both *S. hyicus* subspecies. They differ in several properties including besides the BA and PBP patterns, hyaluronidase and hydrolysis of Tween 80 (Devriese *et al.*, 1978), menaquinone composition (Nahaie *et al.*, 1984), pathogenicity for piglets (Devriese & Oeding, 1975), and susceptibility to H phages (Hájek & Horák, 1981). Moreover, one or more of the following traits are often, but not always, present in only one of the subspecies: coagulase, staphylokinase, pigmentation, and production of acid from maltose, mannitol and turanose under aerobic conditions. The two subspecies also differ in the amount of heat-stable nuclease produced (Devriese *et al.*, 1978). This number of differences between the subspecies raises doubts about the correctness of their taxonomic position. This question was also emphasized by numerical taxonomy which separated the subspecies at 67% similarity (Hájek & Schindler, 1981). On the other hand, a DNA-DNA hybridization study of the *S. hyicus* subsp. *hyicus* and *S. hyicus* subsp. *chromogenes* strains provided homology values ranging from 30 to 55%, which does indicate a relationship at a subspecies level (Devriese *et al.*, 1978). The solution of this taxonomic problem requires further study.

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