Additional Differentiating Characters of the Two Subspecies of Staphylococcus hyicus

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(Received 31 December 1984)

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 79000) while the S. hyicus subsp. chromogenes strains had three distinct PBPs (mol. wts 84000, 82000 and 79000).

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., 1978a, b) and animal (Varaldo et al. 1978b) origin. The pattern of penicillin-binding proteins (PBPs), which are bacterial plasma membrane proteins that specifically and covalently bind β-lactam antibiotics,
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., 1978a, b). The BA substrates, the test media, and the BA estimation were as described previously (Varaldo et al., 1978a; 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 100000 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 SDE, 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.
Staphylococcus hyicus subspecies

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

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

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

<table>
<thead>
<tr>
<th>Species or subspecies</th>
<th>BA on the test media:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TP1 TP2 TP2P T0 T1 T3 B15TP1 a61TP2</td>
</tr>
<tr>
<td>S. hyicus subsp. hyicus</td>
<td>+ + + - + + + ± - - -</td>
</tr>
<tr>
<td>S. hyicus subsp. chromogenes</td>
<td>+ + ± - + + + ± - - -</td>
</tr>
<tr>
<td>S. aureus*</td>
<td>+ + + - + + + ± - - -</td>
</tr>
<tr>
<td>S. intermedius*</td>
<td>+ + + - + + + ± - - -</td>
</tr>
</tbody>
</table>

* According to Varaldo et al. (1978b, 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 79000. By contrast, the strains of S. hyicus subsp. chromogenes shared a PBP pattern characterized by three distinct bands with molecular weights of 84000, 82000 and 79000. 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., 1978a, 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., 1978a), 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
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 (Nahai et al., 1984), pathogenicity for piglets (Devriese & Oeding, 1975), and susceptibility to H phages (Hajek & Horak, 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, mannotol 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 (Hajek & 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.

The authors are greatly indebted to Dr L. A. Devriese (Gent, Belgium) and Dr D. Hajsig (Zagreb, Yugoslavia) for kindly providing the S. hyicus strains. Thanks are also due to I. De Mandina for his skilful technical assistance.

This work was partially supported by grants 83.00696.52 and 84.02042.52 from the Consiglio Nazionale delle Ricerche.

REFERENCES


