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Rapid Test for the Serological Separation of Staphylococci from Micrococci

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A simple test for the serological separation of staphylococci from micrococci is described, which is based on the quite different cell wall peptidoglycan structures of these two genera. Antisera to (pentaglycyl-ε-amino-*n*-hexanoic acid)$_{20}$-albumin agglutinated without exception all staphylococci and gave no positive reaction with micrococci or other bacterial cells. To obtain a good reaction, it was necessary to extract the cells with hot trichloroacetic acid for 30 min. Antisera to (tri-L-alanyl-ε-amino-*n*-hexanoic acid)$_{22}$-albumin reacted strongly with micrococci containing oligo-L-alanine bridges in their peptidoglycan, but did not agglutinate staphylococci or other bacteria lacking alanine interpeptide bridges.

The most widely used routine test for identifying staphylococci is based on their ability to produce acid from glucose when incubated under anaerobic conditions, whereas micrococci lack this ability (1, 10, 11). This oxidation/fermentation test has been a point of controversy for many years, because it does not always provide clear results. Even complete misclassifications may occur, since some micrococci (in particular, strains of *Micrococcus kristinae*) may produce acid under anaerobic conditions like staphylococci, and, moreover, certain strains of staphylococci produce only small amounts of acid when incubated anaerobically or even fail to grow under anaerobic conditions (2, 3, 6, 7). In a very recent study, we reported on specific antibodies to staphylococcal and to micrococcal cell wall peptidoglycans (P. H. Seidl and K. H. Schleifer, Arch. Mikrobiol., in press). The present paper describes the serological differentiation of whole cells of staphylococci or micrococci, using these specific antibodies after appropriate extraction procedures of bacterial cells.

MATERIALS AND METHODS

Bacterial strains. The following bacterial strains were used in the serological tests: *Staphylococcus aureus* ATCC 12600 and ATCC 12601 (American Type Culture Collection, Rockville, Md.); *S. aureus* Copenhagen (J. M. Ghuysen, University of Liège, Liège, Belgium); *S. aureus* H (J. Baddiley, University of Newcastle upon Tyne, Newcastle upon Tyne, England); *S. capitis* ATCC 27840; *S. cohnii* DSM 20260 (Deutsche Sammlung für Mikroorganismen, Munich, West Germany); *S. epidermidis* ATCC 14990; *S. haemolyticus* DSM 20264; *S. hominis* ATCC 27844; *S. saprophyticus* CCM 883 (Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia); *S. sciuri* ATCC 29062; *S. simulans* ATCC 27848; *Staphylococcus* sp. CCM 2495; *S. xylosus* DSM 20266; *S. warneri* ATCC 27836; *Micrococcus kristinae* ATCC 27570; *M. luteus* CCM 169; *M. roseus* H 15 (W. Back, Technical University, Munich, West Germany); *M. varians* CCM 884; *Bacillus subtilis* W 23 (A. R. Archibald, University of Newcastle-upon-Tyne).

Medium and growth conditions. All strains were grown in tubes containing 10 ml of the following medium: 1.5% yeast extract (Difco)-1% peptone from casein (Difco)-0.5% glucose-0.5% NaCl, pH 7.2. The tubes were incubated at 30°C in a water bath shaker for 15 h.

Extraction procedure of whole cells. Cells grown overnight (in 10 ml of medium) were harvested by 10 min of centrifugation at 4,000 × *g*, and the sediment was suspended in 1.5 ml of saline and centrifuged for 2 min with a microcentrifuge (Eppendorf 3200). All the following procedures were performed in the same centrifuge tubes. The sediment was suspended in 1.5 ml of 10% trichloroacetic acid, incubated for 30 min at 100°C, and again centrifuged for 2 min. The sediment was washed three times with 1.5 ml of distilled water and suspended in 1.5 ml of a 0.1 M phosphate buffer, pH 7.8, containing 20 mg of trypsin per 100 ml, and the tubes were then incubated at 37°C for 1 h. The tubes were then centrifuged again and washed twice with distilled water, and the sediment was suspended in 10 ml of borate-NaCl buffer, pH 8.2. One milliliter of a 1:100 dilution of the suspension was applied in latex agglutination.

Antisera. Antisera with specificity to oligoglycine peptides and to oligo-L-alanine peptides were obtained by immunizing rabbits with the synthetic peptidyl-protein antigens (pentaglycyl-ε-amino-*n*-hexanoic acid)$_{20}$-albumin [(pentaglycyl-ε-Ahx)$_{20}$-albumin] and (tri-L-alanyl-ε-Ahx)$_{22}$-albumin, respectively, using the immunization schedule given by Schleifer and Seidl (8). The antigens were synthesized as described in detail by Schechter et al. (4).

Latex agglutination. Agglutination was performed as previously described in detail (9). For borate-NaCl buffer (pH 8.2), NaCl, 850 mg-0.1 M boric

acid, 50 ml–0.1 N NaOH, 5.9 ml was made up to 100 ml with water, and the pH was adjusted to 8.2. For latex stock solution, 2 ml of a latex suspension (Difco) was mixed with 20 ml of water and centrifuged for 3 min at 2,500 rpm, and the supernatant was filtered through Whatman filter no. 40. The filtrate was used as latex stock solution. For immunoglobulin stock solution, a 0.5% solution of isolated immunoglobulin was prepared in borate-NaCl buffer, pH 8.2. For peptidoglycan solution, peptidoglycan stock solutions (10 μg/ml) were diluted geometrically with borate buffer. The test was performed as follows. A mixture of 0.1 ml of latex solution, 0.5 ml of immunoglobulin stock solution, and 9.4 ml of borate buffer was added to 1.0 ml of every peptidoglycan dilution. The reaction mixture was incubated for 2 h at 56°C, and the results were read after 12 h at 4°C.

RESULTS

Specific antibodies were raised by immunizing rabbits with the synthetic peptidyl-protein conjugates (pentaglycyl-ε-Ahx)$_{20}$-albumin or (tri-L-alanyl-ε-Ahx)$_{22}$-albumin (Seidl and Schleifer, Arch. Mikrobiol., in press). The specificities of antisera to these peptidyl-protein conjugates are summarized in Table 1. All staphylococcal peptidoglycans, including those with a serine or an alanine residue incorporated into the oligoglycine-interpeptide bridge, agglutinated strongly with antisera to (pentaglycyl-ε-Ahx)$_{20}$-albumin. The reaction with antisera to (tri-L-alanyl-ε-Ahx)$_{22}$-albumin was negligible. On the contrary, peptidoglycans with an interpeptide bridge composed of three to four L-alanine molecules gave strong reactions with antisera to (tri-L-alanyl-ε-Ahx)$_{22}$-albumin but failed completely to agglutinate with antisera to (pentaglycyl-ε-Ahx)$_{20}$-albumin. Peptidoglycans without an oligoglycine or an oligo-L-alanine interpeptide bridge, used as a "negative control," did not react with either antiserum. The antisera to the synthetic antigens thus allowed the specific detection of staphylococcal peptidoglycans and the clear separation of staphylococcal from micrococcal peptidoglycans.

Application of these antisera to the serological detection of the peptidoglycan type in whole bacterial cells was disappointing, however (Table 2, column a). The titers obtained in the agglutination reaction between whole bacterial cells and antisera to (pentaglycyl-ε-Ahx)$_{20}$-albumin or (tri-L-alanyl-ε-Ahx)$_{22}$-albumin were only very weak or even negative. These negative or weak agglutination reactions were presumably due to other cell wall components (polysaccharides, teichoic acids, proteins) that sterically hindered the reaction between antibodies and peptidoglycan. We therefore developed a rapid procedure for the extraction of whole cells and subsequent determination of agglutination titers with specific "peptidoglycan-type antisera" (compare Materials and Methods). The results are summarized in Table 2. As can be seen from the Table, all staphylococcal cells tested could be specifically detected with antisera to (pentaglycyl-ε-Ahx)$_{20}$-albumin with titers from 1:32 to 1:64, whereas the micrococci with interpeptide bridges composed of three to four L-alanine residues gave, without exception, specific reactions with antisera to (tri-L-alanyl-ε-Ahx)$_{22}$-albumin (titers from 1:8 to 1:32). The titers obtained in the agglutination reaction with isolated peptidoglycans are higher than those yielded with extracted whole cells. Agglutination titers may be increased by prolonged extraction times (unpublished). We found, however, that the extraction times applied were sufficient in all cases hitherto tested.

DISCUSSION

Previous studies demonstrated that staphylococci can be separated from micrococci by their quite different peptidoglycan types. Peptidoglycans with a high content of glycine are typical for staphylococci. Up to now, three different peptidoglycan types have been detected in the genus *Staphylococcus* (5, 7). Strains belonging to *S. aureus* contain a peptidoglycan cross-

TABLE 1. *Latex agglutination of antisera to (pentaglycyl-ε-Ahx)$_{20}$-albumin [(Gly$_5$-ε-Ahx)$_{20}$-albumin] and to (tri-L-alanyl-ε-Ahx)$_{22}$-albumin [(L-Ala$_3$-ε-Ahx)$_{22}$-albumin] with staphylococcal or micrococcal peptidoglycans*

Peptidoglycan type	Latex agglutination titer with antiserum to:		No. of strains tested
	(Pentaglycyl-ε-Ahx)$_{20}$-albumin	(tri-L-alanyl-ε-Ahx)$_{22}$-albumin	
Gly$_{5-6}$-interpeptide bridge (e.g., *S. aureus*)	1:512–1:1,024	1:8	6
Gly$_4$, L-serine$_{0.5-1.5}$-interpeptide bridge (e.g., *S. epidermidis*, *S. saprophyticus*)	1:256–1:512	1:4–1:8	10
Gly$_4$-L-Ala-interpeptide bridge (e.g., *S. lactis* I 3 (= *S. sciuri*)	1:512	1:4	1
L-Ala$_{3-4}$-interpeptide bridge (e.g., *M. roseus*, *M. varians*)	Negative	1:1,024–1:2,048	8
L-Lys directly cross-linked (e.g., *B. subtilis*)	Negative	Negative–1:4	3

TABLE 2. *Influence of different extraction procedures on serological detection of the peptidoglycan type in whole bacterial cells*

Bacterial strains	Latex agglutination titer for peptidoglycan type by procedure[a]:							
	a		b		c		d	
	Gly_5	Ala_3	Gly_5	Ala_3	Gly_5	Ala_3	Gly_5	Ala_3
S. aureus ATCC 12600	1:8	0	1:16	0	1:32	0	1:64	0
S. aureus ATCC 12601	1:8	0	1:16	0	1:32	0	1:64	0
S. aureus Copenhagen	1:4	0	1:8	0	1:16	0	1:32	0
S. aureus H	1:8	0	1:16	0	1:32	0	1:64	0
S. capitis ATCC 27840	1:4	0	1:16	0	1:32	0	1:64	0
S. cohnii DSM 20260	1:2	0	1:8	0	1:16	0	1:32	0
S. epidermidis ATCC 14990	1:4	0	1:16	0	1:32	0	1:32	0
S. haemolyticus DSM 20264	1:4	0	1:8	0	1:16	0	1:16	0
S. hominis ATCC 27844	1:4	0	1:8	0	1:32	0	1:64	0
S. saprophyticus CCM 883	1:2	0	1:8	0	1:16	0	1:32	0
S. sciuri ATCC 29062	1:2	0	1:8	0	1:32	0	1:64	0
S. simulans ATCC 27848	1:2	0	1:8	0	1:16	0	1:64	0
Staphylococcus sp. CCM 2495	0	0	1:8	0	1:32	0	1:64	0
S. xylosus DSM 20266	1:4	0	1:8	0	1:16	0	1:32	0
S. warneri ATCC 27836	1:4	0	1:8	0	1:16	0	1:32	0
M. varians CCM 884	0	1:2	0	1:4	0	1:8	0	1:16
M. kristinae ATCC 27570	0	1:4	0	1:8	0	1:16	0	1:16
M. roseus H 15	0	0	0	1:8	0	1:32	0	1:64
B. subtilis W 23	0	0	0	0	0	0	0	0

[a] Gly_5, Antiserum to (pentaglycyl-ε-Ahx)$_{20}$-albumin; Ala_3, antiserum to (trialanyl-ε-Ahx)$_{22}$-albumin. a, Untreated cells; b, cells treated with 10% trichloroacetic acid at 100°C for 15 min; c, cells treated with 10% trichloroacetic acid at 100°C for 30 min; d, cells treated with 10% trichloroacetic acid for 30 min and incubated with trypsin for 1 h.

linked by penta- or hexaglycine bridges. The peptidoglycans of *S. epidermidis* strains contain also pentaglycine or hexaglycine interpeptide bridges, but usually 15 to 30% of the glycine residues are replaced by L-serine. A few other coagulase-negative strains (*S. sciuri*) reveal a peptidoglycan type in which the interpeptide bridge consists of a tetraglycyl-L-alanine peptide. It should be pointed out that all staphylococci, without exception, contain these oligoglycine peptides. On the other hand, the peptidoglycan interpeptide bridge of many micrococci (e.g., *M. roseus, M. varians*) is built up of three to four L-alanine residues. Analysis of peptidoglycan composition allows, therefore, the clear separation of staphylococci from micrococci.

The chemical determination of the peptidoglycan structure is, however, laborious and time consuming. Although a rapid procedure for establishing the peptidoglycan type of gram-positive organisms has been introduced (5), the chemical method is not suitable for application in rapidly screening numerous bacterial samples in the routine laboratory.

Synthetic peptidyl-protein antigens carrying peptide determinants with structural similarity to the interpeptide bridge of gram-positive cocci are a convenient tool to obtain specific antibodies to different peptidoglycans (9; Seidl and Schleifer, Arch. Mikrobiol., in press). Since the primary structure of peptidoglycan has proved to be a valuable chemotaxonomic criterion for identification and differentiation of gram-positive bacteria (5), specific antisera to particular interpeptide bridges may be a general approach for serological detection of bacteria on the basis of their peptidoglycan types.

In the present report, we have described the serological separation of staphylococci from micrococci. It should once more be kept in mind that all staphylococci, and only staphylococci, reveal interpeptide bridges composed of four to six glycine residues. Specific antisera to (pentaglycyl-ε-Ahx)$_{20}$-albumin should therefore allow, without exception, the specific detection of staphylococci. In any case, the serological separation of staphylococci from micrococci by using these "anti-staphylococcal sera" seems to be more reliable than the classical oxidation/fermentation test. Moreover, it should be pointed out that no preceding purification step is necessary. Because of the characteristic occurrence of oligoglycine interpeptide bridges among staphylococci, the antisera to (pentaglycyl-ε-Ahx)$_{20}$-

albumin allow the specific detection of staphylococci even in a mixture of bacteria.

The described screening test is very simple. Only 10 ml of a culture grown overnight is necessary. The test may be performed with simple equipment (microcentrifuge, water bath, tubes, pipettes) that is available in every laboratory. Moreover, the presented results have revealed that the serological test is highly specific. The sensitivity of the test was found to be in the range of 10^6 to 10^7 cells/ml.

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LITERATURE CITED

1. **Baird-Parker, A. C.** 1963. A classification of micrococci and staphylococci based on physiological and biochemical tests. J. Gen. Microbiol. **30**:409–427.
2. **Evans, J. B., and W. E. Kloos.** 1972. Use of shake cultures in a semisolid thioglycolate medium for differentiating staphylococci from micrococci. Appl. Microbiol. **23**:326–331.
3. **Kloos, W. E., and K. H. Schleifer.** 1975. Isolation and characterization of staphylococci from human skin. II. Descriptions of four new species: *Staphylococcus warneri, Staphylococcus capitis, Staphylococcus hominis,* and *Staphylococcus simulans.* Int. J. Syst. Bacteriol. **25**:62–79.
4. **Schechter, B., I. Schechter, and M. Sela.** 1970. Antibody combining sites to a series of peptide determinants of increasing size and defined structure. J. Biol. Chem. **245**:1438–1447.
5. **Schleifer, K. H., and O. Kandler.** 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. **36**:407–477.
6. **Schleifer, K. H., and W. E. Kloos.** 1975. Isolation and characterization of staphylococci from human skin. I. Amended descriptions of *Staphylococcus epidermidis* and *Staphylococcus saprophyticus,* and descriptions of three new species: *Staphylococcus cohnii, Staphylococcus haemolyticus* and *Staphylococcus xylosus.* Int. J. Syst. Bacteriol. **25**:50–61.
7. **Schleifer, K. H., and W. E. Kloos.** 1976. Separation of staphylococci from micrococci. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Suppl. **5**:1–9.
8. **Schleifer, K. H., and P. H. Seidl.** 1974. The immunochemistry of peptidoglycan. Antibodies against a synthetic immunogen cross-reacting with peptidoglycan. Eur. J. Biochem. **43**:509–519.
9. **Seidl, P. H., and K. H. Schleifer.** 1977. The immunochemistry of peptidoglycan. Antibodies against a synthetic immunogen cross-reacting with an interpeptide bridge of peptidoglycan. Eur. J. Biochem. **74**:353–363.
10. **Subcommittee on Taxonomy of Staphylococci and Micrococci.** 1965. Recommendations. Int. Bull. Bacteriol. Nomen. Taxon. **15**:109–110.
11. **Subcommittee on Taxonomy of Staphylococci and Micrococci.** 1971. Minutes of meeting 2 and 3 April 1968. Int. J. Syst. Bacteriol. **21**:161–163.