Grouping of Staphylococci on the Basis of Their Bacteriolytic-Activity Patterns: a New Approach to the Taxonomy of the *Micrococcaceae*

I. Identification of Six Different "Lyogroups"

PIETRO E. VARALDO, GIUSEPPE SATTA, GRAZIA GRAZI, AND CARMINE A. ROMANZI

Istituto di Microbiologia dell'Università di Genova, Genova, Italy

As a further development of our previous finding that heterogeneous bacteriolytic activities are produced by virtually all staphylococcal strains, several different conditions were tested for their selective and differentiating interferences with the bacteriolytic activity of some staphylococcal strains. Such a study has indicated the possibility of resolving different patterns of lytic activity among staphylococci and has resulted in the preparation of an assay system, composed of eight testing media, for lytic enzymes. A total of 1,054 staphylococcal strains has been tested on these media and found to fall into six "lyogroups," each characterized by a specific pattern of bacteriolytic activity. The novelty and taxonomic relevance of analyzing the bacteriolytic activity of staphylococci and the possibility of extending the proposed method to bacteria other than staphylococci are discussed.

There is not yet general agreement upon both the definition of the genus *Staphylococcus*, in particular its separation from the genus *Micrococcus*, and its subdivision into species or biotypes. Only minor taxonomic problems exist in dealing with coagulase-positive staphylococci. *Staphylococcus aureus* is, in fact, unanimously considered a well-defined and homogeneous species, at least as far as strains of human origin are concerned (3, 5, 8). Most of the problems are concerned with coagulase-negative staphylococci, for these strains are rather heterogeneous, and the schemes proposed for their classification and identification only partially coincide (1, 4, 5, 9, 11, 16, 17).

The discovery of some new physiological property that varies with the different groups of staphylococci would be helpful in resolving these problems.

We have recently shown (14) that under suitable conditions virtually all staphylococci produce an easily detectable bacteriolytic activity. We also found that the optimal conditions for expressing bacteriolytic activity differ not only between coagulase-positive and coagulase-negative staphylococci, but also between different coagulase-negative strains. Therefore, bacteriolytic activity seemed to us to be a good candidate for a characteristic of high taxonomic value.

To test for such a possibility, we studied the bacteriolytic activity of 1,054 staphylococci under various conditions of growth. As a result, we were able to subdivide these strains into six

different groups, which we have named "lyogroups" (λύειν = to lyse), the members of each group producing a particular type of lytic activity. It will be shown in an accompanying paper (18) that strains sharing a particular bacteriolytic activity also share a great majority of other biochemical and physiological characters.

MATERIALS AND METHODS

Bacterial strains. A total of 1,054 staphylococci from humans were examined in this study. Most were isolated from clinical materials—blood, urine, sputum, pus, throat swab, etc.—examined in the Clinical Bacteriology Laboratory of the Institute of Microbiology of the University of Genoa Medical School. Some strains were isolated from the skin, nose, and throat of healthy volunteers. All strains were gram-positive and catalase-positive cocci, and all were recognized as belonging to the genus *Staphylococcus* (2, 7, 15). In the present report, these staphylococci were characterized exclusively by their bacteriolytic activity; their characterization and identification according to conventional criteria are reported in the accompanying paper (18).

Substrates for bacteriolytic activity. Heat-killed cells of a large number of strains were tested as substrates for bacteriolytic activity. Except for *Micrococcus luteus* ATCC 4698, all of the strains were from the collection of our institute.

A heat-killed cell suspension of *M. luteus* was prepared and added to the melted media as described previously (14). The same procedure was employed with the other strains tested as substrates, the only modifications being in the temperature and time of incubation, as needed, to obtain optimal growth.

Search for conditions affecting the bacteriolytic activities of staphylococci. Twenty-two strains chosen at random were used to evaluate the effect of a variety of conditions on bacteriolytic activity. A chemically defined medium (2% tryptone [Difco], 0.2% glucose, 0.5% NaCl, 1.5% agar [pH 6.9]; *M. luteus* as substrate) was used as the reference condition and as the basal medium for testing the various conditions.

Media. All of the media of the assay system by which the bacteriolytic activities of the strains studied were investigated were prepared from one of the following basal media: TP1-base medium (tryptose phosphate broth [Difco] supplemented with 1.5% agar, pH 6.9); TP2-base medium (tryptose phosphate broth [Difco] supplemented with 0.2% yeast extract, 1.7% NaCl, and 0.9% agar [pH 6.9]); and T-base medium (1% tryptone [Difco], 0.05% glucose, 1.5% agar [pH 6.9]). Further details regarding these media are given below in Results.

Inoculation of the media. Inoculation of the media was performed by a multiple inoculating device described previously (14).

Evaluation of bacteriolytic activity. The inoculated media were incubated for 24 h at 37°C and then maintained for 12 h at room temperature. Zones of transparency around the spots of growth indicated lysis of substrate by the growing strains. Variability in the diameters of the spots of growth was observed, however, so that the distance between the edge of the spot and the edge of the zone of transparency, rather than the diameter of the entire zone of transparency, was considered more indicative of the degree of bacteriolytic activity. The results were scored by the following scheme: +++, very large zone of transparency, exceeding the spot of bacterial growth by more than 6 mm; ++, large zone of transparency, exceeding the spot of bacterial growth by 3 to 6 mm; +, moderate zone of transparency, exceeding the spot of bacterial growth by 1 to 3 mm; ±, small zone of transparency, exceeding the spot of bacterial growth by less than 1 mm; —, no detectable zone of transparency. When the determinations of the above-mentioned distances were doubtful owing to a particularly indistinct outline of the zone of transparency, the lower score was, as a rule, used.

RESULTS

Selection of conditions influencing the bacteriolytic activities of staphylococci. The bacteriolytic activities of the 22 staphylococci chosen at random were tested under many conditions to determine which conditions, if any, were useful in distinguishing and identifying those activities.

The conditions tested, either singly or in combination, were: (i) concentrations of the reference—medium components, including tryptone, glucose, NaCl, and agar; (ii) physical conditions (pH of the medium, growth temperature); (iii) addition to the reference medium (deprived of NaCl) of a variety of inorganic salts at different concentrations; (iv) addition to the reference medium of particular substances (e.g., phenol-

phthalein diphosphate, ethylenediaminetetraacetate, sodium lauryl sulfate, sodium deoxycholate) at different concentrations; (v) replacing *M. luteus* with other substrates (heat-killed cells of strains from the genera *Micrococcus*, *Staphylococcus*, *Streptococcus*, *Bacillus*, *Escherichia*, *Klebsiella*, *Proteus*, *Acinetobacter*, *Pseudomonas*, and *Candida* were tested, both in the presence of 0.5 and of 2.2% NaCl); and (vi) other basal media.

Several of the conditions tested were found to selectively interfere with the bacteriolytic activities of some strains. However, some of the conditions, e.g., different salts or different substrates, produced results that were equivalent to each other. Among the conditions producing a differentiating effect, only those not markedly inhibiting cell growth and only one of two or more conditions giving the same result were employed in preparing a set of testing media. Some of the conditions not used, however, might prove useful for possible further modifications of the set of media and for extending this same taxonomic approach to bacteria other than staphylococci.

Choice of an assay system. According to the above-mentioned considerations, a set of 16 testing media was prepared (Table 1). A total of 110 strains was tested on these media to check the possibility of recognizing different groups of strains on the basis of their patterns of lytic activity. At least four different main patterns could be demonstrated (Table 2), and over 90% of the strains tested could be placed in one of these four groups.

To check the stability and reproducibility of the patterns of lytic activity evidenced, all of the 110 above-mentioned strains were subcultured 12 times. The lytic-activity patterns of three different colonies from the last subculture of each strain were analyzed again on the set of 16 media. The same pattern exhibited by the original culture was exhibited by all of the colonies of each strain.

The possibility of recognizing different staphylococcal groups on the basis of lytic activity thus being established, 1,054 strains were tested both to confirm the validity of our system and to identify the major patterns of lytic activity within the genus *Staphylococcus*. Owing to the high number of strains to be tested, only 8 of the 16 media were used: the three media (TP1, TP2, and TP2P) essential for identifying the four above-mentioned main patterns and five other media (T0, T1, T3, B15TP1, and a61TP2) which seemed particularly promising.

Some of the main features of the eight media selected to compose the definitive assay system used in this study are as follows.

TABLE 1. *Compositions of the 16 test media*

Medium	Base medium	Substances added	Substrate
TP1	TP1-base		<i>Micrococcus luteus</i> ATCC 4698
TP1D	TP1-base	0.01% Sodium deoxycholate	<i>M. luteus</i> ATCC 4698
TP2	TP2-base		<i>M. luteus</i> ATCC 4698
TP2D	TP2-base	0.01% Sodium deoxycholate	<i>M. luteus</i> ATCC 4698
TP2P	TP2-base	0.02% Phenolphthalein diphosphate (pentasodium salt)	<i>M. luteus</i> ATCC 4698
TP2S	TP2-base	0.005 M sodium lauryl sulfate	<i>M. luteus</i> ATCC 4698
TP2E	TP2-base	0.01 M ethylenediaminetetraacetate (disodium salt)	<i>M. luteus</i> ATCC 4698
T0	T-base		<i>M. luteus</i> ATCC 4698
T1	T-base	0.75% Sodium chloride	<i>M. luteus</i> ATCC 4698
T2	T-base	2.25% Sodium chloride	<i>M. luteus</i> ATCC 4698
T3	T-base	4% Sodium chloride	<i>M. luteus</i> ATCC 4698
M14TP1	TP1-base		<i>M. varians</i> M14
B15TP1	TP1-base		<i>M. roseus</i> B15
A8TP1	TP1-base		<i>Staphylococcus aureus</i> A8
a61TP2	TP2-base		<i>S. epidermidis</i> a61
A12TP2	TP2-base		<i>Klebsiella pneumoniae</i> A12

TABLE 2. *Bacteriolytic-activity patterns of the four groups recognized from 110 strains of staphylococci studied**

Group	Bacteriolytic activity produced by the staphylococci studied on the test media:															
	TP1	TP1D	TP2	TP2D	TP2P	TP2S	TP2E	T0	T1	T2	T3	M14TP1	B15TP1	A8TP1	a61TP1	A12TP2
A	+++	++	+	±	—	+	+	±	++	+	—	++	+	+	—	—
B	++	+	+	+	+	±	±	+	++	+	—	+	—	+	—	—
C	+	±	++	++	±	+	+	—	±	++	±	±	—	—	+	±
D	—	—	+	+	+	—	—	—	—	±	±	—	—	—	—	—

* Symbols for bacteriolytic activity: —, no detectable zone of transparency; ±, +, ++, +++, different sizes of zones of transparency (see text).

TP1 medium. In the preliminary screening, TP1 medium proved to be equivalent to the reference medium; most strains exhibited variable zones of transparency on this medium whereas some were inactive.

TP2 medium. Although the zones of transparency were variable, they were easily detected and were produced by virtually all of the strains; the zones significantly differed from those produced by the same strains on TP1.

TP2P medium. On TP2P medium, some strains exhibited a zone of transparency similar to that produced on TP2; some showed a decreased zone, and others even lost their bacteriolytic activities.

T0, T1, T3 media. The relationship of bacteriolytic activity to NaCl concentration was investigated using T0, T1, and T3 media; as per our previous finding that the effect of NaCl is stressed and more critical in poor media (14), the T-base medium was intentionally a poor one.

B15TP1 and a61TP2 media. Both B15TP1 and a61TP2 media contained substrates other than *M. luteus*; only a minority of staphylococci produced a zone of transparency on either medium.

Analysis of the patterns of lytic activity

and the grouping of 1,054 staphylococci.

The bacteriolytic activities of the 1,054 strains were tested on the eight above-mentioned assay system media. The different patterns obtained and the number of strains exhibiting the same patterns are presented in Table 3. In this table the different patterns were arranged in an order designed to point out that some differences were slight and negligible whereas others were significant and qualitative. The strains tested could be subdivided into six different groups (herein called "lyogroups") depending on the bacteriolytic pattern exhibited. Fourteen strains could not be included in any of the six lyogroups. This subdivision and the number of strains in each lyogroup are reported in Table 4.

Characterization and identification of the staphylococcal lyogroups. A summary scheme for grouping staphylococci on the basis of their bacteriolytic-activity patterns, as determined by the assay system, is shown in Table 5.

In the 14 strains not included in any of the six lyogroups, some similarities in bacteriolytic-activity patterns have been found which may suggest the occurrence of further and probably less numerous staphylococcal lyogroups.

In conclusion, when a staphylococcal strain is

TABLE 3. *Bacteriolytic-activity patterns of the 1,054 staphylococci tested on the assay-system media^a*

Pattern no.	No. of strains ^c	Bacteriolytic activity produced by the staphylococci studied on the test media:							
		TP1	TP2	TP2P	T0	T1	T3	B15TP1	a61TP2
1	241	+++	+	-	±	++	-	+	-
2	47	+++	+	±	±	++	-	+	-
3	16	+++	+	-	+	++	-	+	-
4	2	+++	+	±	+	++	-	+	-
5	14	+++	+	-	±	++	±	+	-
6	64	+++	++	-	±	++	±	+	-
7	1	+++	+	-	-	++	-	+	-
8	9	+++	+	±	-	++	-	+	-
9	5	++	+	-	±	+	-	+	-
10	10	++	+	±	±	+	-	+	-
11	4	++	+	±	±	+	-	±	-
12	25	++	+	+	+	++	-	-	-
13	7	++	+	+	+	++	±	-	-
14	6	++	+	+	±	++	-	-	-
15	1	++	+	+	±	+	-	-	-
16	3	+	-	-	±	+	-	-	-
17	2	+	-	-	-	+	-	-	-
18	12	+	±	±	++	±	-	-	-
19	5	+	±	±	+	±	-	-	-
20	2	++	±	±	++	+	-	-	-
21	4	+	±	±	+	-	-	-	-
22	1	+	±	±	±	-	-	-	-
23	1	++	+	-	++	++	±	-	-
24	14	+++	+++	+++	+	++	+	-	-
25	4	+++	+++	+++	±	++	+	-	-
26	2	+++	+++	+++	+	++	±	-	-
27	3	+++	+++	++	+	++	+	-	-
28	1	+++	+++	+	+	++	+	-	-
29	2	+++	++	++	+	++	+	-	-
30	3	++	+++	+++	±	+	±	-	-
31	11	++	++	++	±	++	+	-	-
32	2	++	++	+	±	++	+	-	-
33	1	+	++	++	-	++	+	+	-
34	1	+	++	++	-	+	±	±	-
35	1	+	++	++	±	±	±	±	-
36	91	+	+++	±	-	±	++	-	+
37	37	+	+++	-	-	±	++	-	+
38	2	+	+++	+	-	±	++	-	+
39	29	+	+++	±	-	±	+	-	+
40	5	+	+++	±	-	+	++	-	+
41	1	+	+++	±	-	-	++	-	+
42	3	+	+++	-	-	-	++	-	+
43	4	+	+++	-	-	-	+	-	+
44	3	+	+++	±	-	±	++	-	++
45	7	+	+++	-	-	±	++	-	++
46	18	±	+++	±	-	±	++	-	+
47	2	+	+++	±	-	±	++	±	+
48	4	+	+++	-	-	±	++	±	+
49	10	±	++	±	±	±	+	-	+
50	9	±	++	-	-	±	+	-	+
51	1	±	++	-	-	-	+	-	±
52	13	+	+++	+++	-	±	++	-	++
53	1	+	+++	+++	-	±	++	-	++
54	5	+	+++	+++	-	±	+	-	+
55	1	±	++	++	-	±	+	-	+
56	8	+	++	±	-	±	++	-	+
57	1	±	++	±	-	±	++	-	+
58	2	+	++	-	-	±	++	-	+
59	1	±	++	±	-	±	++	-	++
60	4	+	++	++	-	±	++	-	+

TABLE 3. *Continued*

Pattern no.	No. of strains	Bacteriolytic activity produced by the staphylococci studied on the test media:							
		TP1	TP2	TP2P	T0	T1	T3	B15TP1	a61TP2
61	3	+	++	++	—	±	++	—	++
62	2	±	++	++	—	±	++	—	++
63	142	—	+	+	—	—	±	—	—
64	88	—	+	+	—	—	—	—	—
65	4	—	+	+	—	—	+	—	—
66	10	±	+	+	—	±	+	—	—
67	4	±	++	++	—	±	+	—	—
68	2	—	±	±	—	—	±	—	—
69	5	—	±	±	—	—	—	—	—
70	5	—	—	—	—	—	—	—	—

^a Symbols for bacteriolytic activity: —, no detectable zone of transparency; ±, +, ++, +++, different sizes of zones of transparency (see text); *, particularly clear zone of transparency.

TABLE 4. *Lyogroups of the 1,054 staphylococci studied*

Lyogroup	Bacteriolytic patterns ^a	No. of strains
I	1 to 11	413
II	12 to 15	39
III	18 to 22	24
IV	24 to 32	42
V A	36 to 55	246
V B	56 to 62	21
VI	63 to 69	255
Ungrouped strains	16, 17 23 33, 34, 35 70	14

^a The bacteriolytic-pattern numbers refer to those cited in Table 3.

examined for grouping by using the assay system described herein, the following essentials should be taken into consideration: (i) size and features of the zone of transparency produced on TP1; (ii) size and features of the zone of transparency produced on TP2; (iii) comparison between the zones of transparency on TP1 and TP2; (iv) comparison between the zones of transparency on TP2 and TP2P; (v) comparison of the zones of transparency on T0, T1, and T3; (vi) ability to produce a zone of transparency on B15TP1; and (vii) ability to produce a zone of transparency on a61TP2.

DISCUSSION

We have previously shown (14) that not only coagulase-positive but also coagulase-negative staphylococci produce an easily detectable bacteriolytic activity. We were also able to explain the reason for the contrasting results obtained by previous authors with regard to the incidence of bacteriolytic activity produced by coagulase-negative strains: only under suitable conditions

can the bacteriolytic activities of the latter strains be evidenced. Such conditions, on the one hand, are generally different from the optimal conditions for bacteriolytic activity of coagulase-positive strains and, on the other hand, can differ significantly for the various coagulase-negative strains. These findings suggested that the staphylococci are heterogeneous with respect to their bacteriolytic activities.

Therefore we extended our previous approach to the problem by preparing a set of different media for demonstrating possible differences in the bacteriolytic activities of staphylococci. Using these media, we confirmed the putative heterogeneity, and at least six different main patterns of bacteriolytic activity emerged. Consequently, six different staphylococcal lyogroups have been established, each one characterized by the production of a specific bacteriolytic activity.

Obviously it is possible, either by increasing the number of strains tested or by improving the assay system, that additional patterns of lytic activity may be recognized. The 14 strains that could not be placed into any of the six lyogroups may, for instance, lead to the establishment of additional lyogroups.

The positive results obtained with staphylococci of human origin suggest that the assay system described here might also be useful with staphylococci from other sources. Moreover, since members of other bacterial genera have been reported to excrete bacteriolytic enzymes (6, 10, 12, 13, 19), it may prove useful to apply this same approach to them. Furthermore, members of still other genera not believed to produce bacteriolytic activity may show such a property when tested under appropriate conditions, as was the case with most coagulase-negative staphylococci.

The results presented here do not rule out the possibility that the differences observed in the

TABLE 5. *Bacteriolytic-activity patterns of the lyogroups of the staphylococci studied^a*

Lyogroup	Bacteriolytic activity produced by the staphylococci studied on the test media:							
	TP1	TP2	TP2P	T0	T1	T3	B15TP1	a61TP2
I	+++	+	—	±	++	—	+	—
II	++	+	+	+	++	—	—	—
III	+	±	±	++	±	—	—	—
IV	+++	+++	+++ ^b	+	++	+	—	—
V A	+	++*	± ^c	—	±	++	—	+
V B	+	++	± ^c	—	±	++	—	+
VI	—	+	+	—	—	±	—	—

^a Symbols for bacteriolytic activity: —, no detectable zone of transparency; ±, +, ++, +++, different sizes of zones of transparency (see text); *, particularly clear zone of transparency (this property, which is not present in strains of subgroup B, is the basis for differentiating subgroup A strains from those of subgroup B).

^b A minority of strains (about 14%) showed a decreased bacteriolytic activity on TP2P in comparison with that on TP2.

^c A minority of strains (about 8% in subgroup A and 43% in subgroup B) showed on TP2P a bacteriolytic activity identical to that on TP2.

bacteriolytic activities of different strains are due to specific interference with bacteriolytic-enzyme production. However, in another study (P. E. Varaldo, G. Satta, G. Grazi, and C. A. Romanzi, manuscript in preparation), we tested the effect of the conditions of the assay system on the lytic activities of the culture supernatant and of the partially purified enzyme of some representative strains of each lyogroup. We found that the culture supernatants showed exactly the same patterns as those produced by the corresponding strains on solid media. Moreover, the purified enzymes from the strains of different lyogroups have been found to differ from one another in a number of chemical and physical properties. Therefore, the assay system described here allows an easy and rapid identification of each of these different lytic activities, thus providing a new manner of subdividing staphylococci.

As previously pointed out, both the definition and the subdivision of the genus *Staphylococcus* are still under question. Based on the results of this study, we suggest that bacteriolytic activity be exploited as a new criterion in the taxonomy of the *Micrococcaceae*.

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REPRINT REQUESTS

Address reprint requests to: Dr. Pietro E. Varaldo, Istituto di Microbiologia dell'Università di Genova, Viale Benedetto XV, 10, 16132 Genova, Italy.

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