Serological Grouping of Virulent and Avirulent Strains of the Lobster Pathogen *Aerococcus viridans*

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SUMMARY

Virulent strains of *Aerococcus viridans* (formerly *Gaffkya homari*) are the aetiologic agents of gaffkemia, a septicaemic disease of the American lobster (*Homarus americanus*). The virulent and avirulent forms of this bacterium, previously thought to be taxonomically indistinguishable, have been differentiated by serological studies. Antisera were produced in rabbits using autoclaved bacteria as antigens. Reactions were measured by agglutination tests using microtitre techniques and an antigenic scheme was determined. Specific antisera were prepared by absorption and used to determine antigens of strains of *A. viridans* and other Gram-positive cocci. In general, only virulent strains of *A. viridans* have antigen b. Both virulent and avirulent strains possess other antigens also detected in strains of the genus *Staphylococcus*.

INTRODUCTION

From diseased American lobsters (*Homarus americanus*), Snieszko & Taylor (1947) isolated a Gram-positive tetrad-forming coccus for which Hitchner & Snieszko (1947) suggested the name *Gaffkya homari*. The pathogenicity of this bacterium for *H. americanus* was verified by Rabin (1965) and Stewart, Foley & Ackman (1969). The genus name *Gaffkya* was rejected (Editorial Secretary, 1971) as a result of the work of Deibel & Niven (1960), who recommended that the lobster pathogen be included in the genus *Pediococcus*.

Williams, Hirsch & Cowan (1953) recommended a new genus, *Aerococcus*, with the type species *Aerococcus viridans*, to accommodate strains of Gram-positive, tetrad-forming cocci isolated from air (Williams & Hirsch, 1950; Shaw, Stitt & Cowan, 1951). Later, similar bacteria were isolated from other sources (Deibel & Niven, 1960; Clausen, 1964; Mundt, Grahm & McCarty, 1967; Colman, 1967; Kerbaugh & Evans 1968). A number of studies have shown aerococci to resemble the lobster pathogen (Aaronson, 1956; Deibel & Niven, 1960; Whittenbury, 1965; Sakaguchi & Mori, 1969; Evans & Schultes, 1969; Nakel, Ghuyssen & Kandler, 1971; Schultes & Evans, 1971). Kelly & Evans (1974) recommended that the lobster pathogen be included in the genus *Aerococcus* as a variety of *A. viridans*. Puzniak, Chesbro & Evans (1971) found that two strains of *A. viridans* were not pathogenic for the American lobster. Whether the lobster pathogen belongs to the genus *Aerococcus* or the genus *Pediococcus*, or to another genus, remains controversial. However, *Bergey's Manual of Determinative Bacteriology* (1974) incorporates the suggestions of Kelly & Evans (1974) and both virulent and avirulent strains are included as *A. viridans*.

Little serological work has been done with *A. viridans*. Serological studies of *Pediococcus* strains (Gunther & White, 1961; Coster & White, 1964) have shown that avirulent strains of

Table 1. *Source and designation of bacterial strains*

Name (as received)	Strain designation	Source†
<i>Pediococcus homari</i> *	Ph-3 Ph-4, 5, 6, 13, 14, 15, 17, 18, 23, 24, 27, 29, 30, 33, 34, 35, 36, 38, 42, 43 CSB-6, 7, 11	Nierengarten, G. M. Kellogg, S. T. F. Steenbergen
<i>P. pentosacens</i>	8081	ATCC
<i>P. soyaе</i>	13621	ATCC
<i>Gaffkya homari</i> *	Ph-1, 7; Rabin; SAB16; StA8, 11, 13, 14, 15, 16, 17, 18, 19, 39 10400	Stewart, J. E. ATCC
<i>Aerococcus viridans</i> *	MK-3, 32, 53, 55, 83, 95, 132, 146, 165, 188, 201, 767, 1486 FW-77, 80, 82 WC-7, 10; LS-17; 301; 302; 303; C-7 8251	Evans, J. B. Evans, J. B. Evans, J. B. NCTC
<i>A. catalasicus</i>	C-6, 9	Evans, J. B.
<i>Staphylococcus epidermidis</i>	14990	ATCC
<i>Staph. aureus</i>	12600 CN-1	ATCC F. Steenbergen
<i>Streptococcus lactis</i>	19435	ATCC
<i>Strep. faecalis</i>	6057	ATCC
<i>Strep. salivarius</i>	13419	ATCC

* Strains received as *P. homari* and *G. homari* are now considered to be strains of *Aerococcus viridans*.

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A. viridans are distinct from the pediococci, but strains of the lobster pathogen were not included. The purpose of this study was to determine the serological relationships between virulent and avirulent forms of *A. viridans*.

METHODS

Bacteria. These are listed, with the names as received, in Table 1. Stock cultures were maintained at room temperature on a solid medium and subcultured every 10 days.

Media. Liquid medium was BBL Trypticase Soy Broth plus 0.3% (w/v) yeast extract (TSBY). Solid medium was TSBY plus 1.5% (w/v) agar. Media were sterilized by autoclaving at 121 °C for 15 min.

Antiserum production. Avirulent *A. viridans* strains MK-3 and MK-53, and virulent strains Ph-3, Ph-7, Ph-15, Ph-27, Ph-29, Ph-30, WC-7 and StA18 were used to prepare antisera. The bacteria were grown in 300 ml TSBY for 20 h, harvested, washed three times in saline, resuspended in 10 ml saline, and autoclaved for 1 h at 121 °C. These preparations, adjusted to McFarland nephelometer standard number 10, were used to raise antisera in rabbits by standard techniques. The antigen preparations were autoclaved because in this way serum titres were increased at least fourfold relative to those obtained with preparations that were not autoclaved. Absorbed antisera were prepared by the double absorption technique. The serum dilution ratio was 1:2.

Agglutination tests. The antigen for agglutination tests was the same as the injected

Table 2. *Agglutination reactions of absorbed antisera*

Absorbed serum*	Antibodies in serum	Reacting strains									
		MK-3	Ph-15	WC-7	Ph-7	Ph-3	StA18	Ph-27	Ph-29	Ph-30	MK-53
Ph-7(MK-3) Ph-15(Ph-3) Ph-27(Ph-3) Ph-29(Ph-3) Ph-30(Ph-3) StA18(Ph-3) WC-7(Ph-3)	b	—	+	+	+	—	+	+	+	+	—†
MK-3(WC-7) MK-3(Ph-29)	cd	+	—	—	—	+	—	—	—	—	—
Ph-3(WC-7) Ph-3(MK-53)	ce	+	—	—	—	+	—	—	—	—	—
MK-3(WC-7, Ph-3)	d	+	—	—	—	—	—	—	—	—	—
MK-53(Ph-7, Ph-3)	d	+	—	—	—	—	—	—	—	—	—‡
Ph-3(WC-7, MK-3)	e	—	—	—	—	—	—	—	—	—	—
MK-53(Ph-3)††	bd	—§	+	+	+	—	+	+	+	+	+
MK-53(Ph-7, MK-3)	none	—	—	—	—	—	—	—	—	—	—

+, Positive reaction; —, no detectable reaction.

* The absorbed serum is denoted as the original serum, with the strain used for absorption in parentheses. For example, WC-7(Ph-3) is antiserum to strain WC-7 that has been absorbed with strain Ph-3.

† MK-53 has a masked b antigen; see Discussion.

‡ MK-53 has a partially masked d antigen; see Discussion.

§ MK-3 reacts poorly with heterologous antisera. Reaction with this absorbed serum is too low to detect.

antigen, but was adjusted to McFarland nephelometer standard number 3. Tests were conducted using microtitre equipment (Cooke Engineering Co.) and clear U-bottom dilution plates (Linbro Chemical Co.). Plates were incubated for 2 h at 50 °C, and then overnight at 4 °C, before examination under a dissecting microscope at $\times 10$ magnification. Each titration was repeated at least twice and a control containing equal volumes of saline and bacterial suspension was included with each titration.

All bacteria (Table 1) were surveyed using specific absorbed antisera, diluted 1:32 in saline to minimize non-specific agglutination. Reactions were read as either positive or negative.

RESULTS AND DISCUSSION

Pre-immunization sera generally had titres of less than 4, although a few had titres of 8. A notable exception was that most of these sera had a titre of 32 to 64 against strain Ph-3.

The 10 *Aerococcus* strains reacted with all 10 unabsorbed antisera, although some titres were very low. These reactions could be due to a common antigen or to nonspecific cross-reactions. We favour the former and propose a common antigen a.

When antisera to strains Ph-7, Ph-15, Ph-27, Ph-29, Ph-30, StA18 and WC-7 were absorbed with strains MK-3 or Ph-3, positive reactions still occurred with all strains tested except MK-3, MK-53 and Ph-3 (Table 2). This indicates the presence of a common antigen, b, in these strains. The following absorbed antisera gave negative results with all strains (the absorbed antiserum is denoted as the original serum, with the strain used for absorption in parentheses): Ph-15(Ph-7), Ph-7(StA18), WC-7(Ph-7), StA18(WC-7), StA18(Ph-29) and Ph-29(WC-7). Apparently, antibodies a and b have been removed, and no other antibodies are detected.

Table 3. *Agglutination reactions of specific absorbed antisera*

Strain	Antigens	Virulence*	Strain	Antigens	Virulence*
<i>A. viridans</i>			CSB-6	c	+
Ph-1	b c† d e	+	CSB-7	d	+
Ph-3‡	c e	+	CSB-11	c	+
Ph-4	b	+	MK-3‡	c d	—
Ph-5	b	+	MK-32	b	NT
Ph-6	b	+	MK-53‡	(b)§ (d)§	—
Ph-7‡	b	+	MK-55	d	±
Ph-13	b	+	MK-83		±
Ph-14	b	+	MK-95		±
Ph-15‡	b	+	MK-132		—
Ph-17	b	+	MK-146		—
Ph-18	b	+	MK-165	c† d e	±
Ph-23	b	+	MK-188	c d	—
Ph-24	b	+	MK-201	b	±
Ph-27‡	b	+	MK-767	b	±
Ph-29‡	b	+	MK-1486		±
Ph-30‡	b	+	FW-77		±
Ph-33	b	+	FW-80	c d	—
Ph-34	b	+	FW-82		±
Ph-35	b	+	WC-7‡	b	+
Ph-36	b	+	WC-10	b	+
Ph-38	b	+	LS-17		NT
Ph-42	b d	+	301		NT
Ph-43	b	+	302		NT
SAB16		+	303		NT
Rabin	b	+	8251	b c d	NT
Sta8	b d	+	C-7	b c d	NT
Sta11	b	+	<i>A. catalasicus</i> C-6	c d	NT
Sta13	b	+	<i>A. catalasicus</i> C-9	c d	NT
Sta14	b	+	<i>P. pentosacens</i> 8081		NT
Sta15	b	+	<i>P. soyae</i> 13621		NT
Sta16	b	+	<i>Staph. epidermidis</i> 14990	c	NT
Sta17	b d e	+	<i>Staph. aureus</i> 12600	c† d e	NT
Sta18‡	b	+	<i>Staph. aureus</i> CN-1	c† d e	NT
Sta19	b	+	<i>Strep. lactis</i> 19435		NT
Sta39	b c d e	+	<i>Strep. faecalis</i> 6057		NT
10400		NT	<i>Strep. salivarius</i> 13419		NT

* Virulence for *H. americanus*: +, virulent; ±, intermediate virulence (Cherry, 1974); —, avirulent; NT, not tested.

† Antigen c in the presence of both d and e.

‡ Strains to which antisera were prepared.

§ Present as an immunogen, not an antigen.

The reactions of absorbed antisera MK-3(WC-7), MK-3(Ph-29), Ph-3(WC-7) and Ph-3(MK-53) indicate that strains MK-3 and Ph-3 have an antigen in common, antigen c (Table 2). The reaction of absorbed antiserum MK-3(WC-7, Ph-3) indicates that MK-3 has an antigen not shared by Ph-3, antigen d. The reaction of the absorbed serum MK-53(Ph-7, Ph-3) indicates that MK-53 shares a common antigen with MK-3, antigen d (Table 2). This is discussed later.

Table 3 shows the results of agglutination reactions of antigen-specific antisera with strains of *A. viridans* and representatives of other genera of Gram-positive cocci. Specific antisera could not be made for antigens a and c. Antisera specific for cd or ce were used to determine if, by inference, c was present. Antigen c could not be differentiated if antigens c and e were both present.

Table 4. *Proposed antigenic scheme for Aerococcus viridans*

Strain	Antigens present	Strain	Antigens present
MK-3	a c d	StA18	a b
Ph-15	a b	Ph-27	a b
WC-7	a b	Ph-29	a b
Ph-7	a b	Ph-30	a b
Ph-3	a c e	MK-53	a (b)* (d)*

*Masked antigens.

In some cases, sera taken from rabbits before immunization gave agglutination titres of 32 to 64. These titres indicate nonspecific reactions. Strain Ph-3, which often gave such nonspecific reactions, possesses an antigen e (Table 3), which is shared in common with some strains of *Staph. aureus*. We suggest that the high nonspecific reactions are the result of prior exposure of the rabbits to *Staph. aureus*.

Some anomalous reactions were observed. The antisera prepared against strains MK-53, Ph-7, Ph-15, Ph-27, and Ph-29 gave higher titres with heterologous strains than with their homologous strain. Strain MK-53 was especially poor, giving many negative or prozone reactions and reactions of low titre; however, antisera to strain MK-53 reacted well with most strains. In this study, a distinction is made between an immunogen and an antigen. Immunogenicity refers to the immunizing potential of a substance, whereas antigenicity refers to the reactivity of a substance with antibody. In order to react by agglutination with homologous antibody, an antigenic determinant must be on the outer surface of the bacterium. Certain surface antigens of strains MK-53 are apparently covered, or masked, by other surface structures. When this bacterium is injected into a rabbit, it is likely that partial digestion exposes the masked antigens. Thus, strain MK-53 has good immunogenicity but poor antigenicity.

In rabbits, strain MK-53 induces the production of antibodies to antigens b and d. *In vitro* strain MK-53 does not react with b antisera, and so b is a masked antigen. Strain MK-53 reacts with d antiserum from rabbits immunized with strain MK-53, but not with d antiserum from rabbits immunized with strain MK-3; thus, antigen d is partially masked. The chemical bases for these differences are under investigation. Similar masking phenomena with other strains, such as Ph-15, could account for heterologous titres being greater than homologous titres and for other low or negative results.

The serological survey of some virulent and avirulent strains of *A. viridans* (Table 3) indicates that there is a good but not complete correlation between the presence of the b antigen and virulence in the American lobster (Cherry, 1974). The b antigen is possessed by 41 of the strains tested; of these, 35 are highly virulent for the American lobster, two are intermediate in virulence (Cherry, 1974), one (MK-53) is avirulent and three have not been tested.

These data support the conclusion of others (Aaronson, 1956; Whittenbury, 1965; Evans & Schultes, 1969; Nakel *et al.*, 1971; Schultes & Evans, 1971; Kelly & Evans, 1974) that there is a very close taxonomic relationship between virulent and avirulent strains of *A. viridans*. The presence or absence of the b or 'virulence' antigen would not, in itself, be sufficient to warrant dividing the organisms into two taxonomic groups.

Pediococcus pentosaceus, *P. soyae* and the three species of the genus *Streptococcus* did not react with typing sera (Table 3) indicating that they share no antigens with *A. viridans*. On the other hand, *A. viridans* apparently shares antigens with strains of the genus *Staphylococcus*. The two members of the genus *Staphylococcus* which were studied reacted with all typing antisera except for the b antiserum.

The proposed antigenic scheme for the 10 *A. viridans* strains to which antisera were prepared is shown in Table 4.

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