Base Ratio and Deoxyribonucleic Acid Homology Studies of Six "Staphylococcus aureus" Typing Bacteriophages

MICHAEL W. PARIZA* AND JOHN J. IANDOLO

Division of Biology, Kansas State University, Manhattan, Kansas 66506

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Genetic relatedness among Staphylococcus aureus typing bacteriophages 80, 47, 81, 71, 77, and 187 was investigated by using base ratio determinations and deoxyribonucleic acid (DNA)-DNA hybridization. Guanine/cytosine (G/C) content, as determined by thermal denaturation and chromatographic analysis of the purines released by acid hydrolysis of the DNA, was between 31 and 36%. No pattern correlating G/C content with serological or lytic group was discernible. DNA-DNA hybridization studies indicated high degrees of homology (43% or more) among the genomes of phages in the same serological group. Less homology (29% or less) was observed between the genomes of phages belonging to different serological groups. These findings implied a positive correlation between serological and genetic relatedness.

The biochemical and genetic characteristics of the typing bacteriophages of Staphylococcus aureus are poorly understood. Past investigators (16–19) have predominantly concerned themselves with serological properties and host range rather than with defining the fundamental properties of these phages. Present observations are hampered by the fact that these viruses are subject to lysogenization, host modification, and host range mutation which alter their expression. Furthermore, little is known of the events or conditions controlling these alterations. For any of these questions to be fully understood, detailed studies must be carried out to define many of the physical properties of the viruses and their components. The experiments presented in this communication are intended to provide some of these necessary data. They include an assessment of genetic relatedness as measured by the guanine/cytosine (G/C) contents of the phage deoxyribonucleic acid (DNAs), and by DNA-DNA hybridization.

MATERIALS AND METHODS

Bacteriophage and propagating strains. S. aureus typing bacteriophages 47 (serological group A, lytic group III), 81 (serological group A, lytic group miscellaneous), 71 (serological group B, lytic group II), 80 (serological group B, lytic group 1), 77 (serological group F, lytic group III), and 187 (serological group L, lytic group miscellaneous), and their propagating strains (PS), PS 47, PS 81, PS 71, PS 80, PS 77, and PS 187, were obtained from the Center for Disease Control (CDC), Atlanta, Ga.

Lyophilized PS cultures were reconstituted with 3 ml of sterile trypticase soy broth (TSB). Volumes of 0.5 ml were removed and inoculated into 100 ml of sterile TSB in 300-ml flasks. The flasks were shaken at 37°C until heavy growth was evident. Organisms were then transferred to blood agar slants, incubated for 24 h at 37°C, and stored at 4°C. The slants, which served as inocula for routine PS preparations, were periodically prepared anew from the original vials.

Stock phages were prepared by incubating broth or soft agar PS preparations (10⁶ cells/ml) with appropriate samples of phage from the vials supplied by the CDC (6). The propagating media used were trypticase soy agar (TSA) plus 0.004% CaCl₂, or TSB plus 0.004% CaCl₂ (2, 6).

Production of bacteriophage. Both broth and soft agar methods were employed for phage production. A final concentration of 10⁴ PS cells/ml in logarithmic growth was prepared in 1.5 to 3 liters of TSB plus CaCl₂ or soft (0.5% agar) TSA plus CaCl₂. Sufficient phage were added to produce near-confluent lysis in soft agar (2, 6) or lysis in broth in 4 to 8 h.

 Cultures were incubated at 30°C (phages 71, 81, and 187) or 37°C (phages 47 and 80). After 12 to 18 h of incubation, 0.5 volume of 1 x SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) was added to the soft agar lysate, and the slurry was rotated at 60 rpm for 4 h at 4°C. The liquid phage was removed, and the agar was reextracted with a 0.5 volume of 1 x SSC. The SSC extracts were pooled and centrifuged at 10,000 x g for 10 min at 4°C. Residual agar was removed by vacuum filtration of the clarified fluid through a single thickness of filter paper.

Broth preparations were incubated at 30°C for
phages 71, 77, 81, or 187 cultures) or 37 C (for phage 47 cultures) with constant agitation at 200 rpm on a rotary shaker. When lysis of phage-infected cells was complete, 1 µg of deoxyribonuclease (DNase; Sigma Chemical Co., DN-C) per ml was added to the suspension and incubation was continued at 4 C for 12 h. One-twentieth volume of 20 x SSC was added, and incubation at 4 C was continued for 4 h. Debris was then removed by centrifugation at 10,000 x g for 10 min at 4 C.

For the production of isotopically labeled phage DNA, [methyl-3H]thymidine (Schwarz, 14 to 19 Ci/mm) was added to a final concentration of 1 to 2 µCi/ml to broth or soft agar at the time of phage infection.

The phage was collected by centrifugation of crude clarified lysates (broth or soft agar) at 21,000 rpm in a Beckman type 21 rotor for 100 min at 4 C. The supernatant fluid was discarded, and the phage pellets were suspended in 15 to 30 ml of 1 x SSC by gentle agitation (60 rpm) at 4 C for 12 h. The resuspended phage was centrifuged at 1,000 x g for 5 min at 4 C to sediment debris. The pellet was resuspended in 1 x SSC, and the slurry was centrifuged as above. The supernatant fluids were pooled.

**Bacteriophage purification.** To prepare gradients, stock CsCl (American Potash and Chemical Corp.) solutions with densities of 1.6, 1.5, 1.475, 1.45, 1.4, and 1.35 g/cm3 in 1 x SSC were sequentially added (beginning with the most dense) in 5-ml layers. Phage suspensions in 5 to 10 ml of 1 x SSC were layered onto the gradients and sedimented into a thin band by centrifugation at 20,000 rpm (SW27 rotor) for 8 to 12 h at 4 C. The tubes were punctured and the opalescent phage bands were collected. CsCl was removed by two 12-h periods of dialysis at 4 C against 100 volumes of 1 x SSC.

The lytic spectra of the purified phage preparations were determined by standard methods (2, 6). Contaminating phage levels (measured by lytic spectra) were less than 0.0001%. The reactions obtained with the preparations (Table 1) were all satisfactory and, in most instances, identical to the published values.

**DNA extraction.** DNA was extracted from purified phage by a modification of the method of Thomas and Abelson (25). Neutralized phenol was added to an equal volume of phage (in 1 x SSC) in a 300-ml flask, and the mixture was rotated at 60 rpm at room temperature for 30 min. The phenol-phage slurry was then carefully poured into glass centrifuge tubes and cooled on ice for 5 min. After centrifugation at 750 x g for 5 min, the phenol layer was removed and the aqueous layer was reextracted with fresh phenol for 20 min and then for 10 min. Residual phenol was removed by dialysis against four changes of 1,000 volumes of 1 x SSC.

**Base ratio analyses.** Chemical analysis was performed by a modification of the methods of Broughton et al. (3) and Kirk (9). The methods are based on determination of adenine/guanine ratios after acid hydrolysis to liberate purines. Phage DNA (100 to 200 µg, cold trichloroacetic acid precipitated), suspended in 1 ml of 0.1 N HCl (3), was placed in a boiling-water bath and heated for 80 min (9). After cooling on ice, the hydrolysate was lyophilized to dryness, and dissolved in 20 µlitters of 1.0 N HCl. The solution was then spotted on Whatman no. 1 chromatographic paper (46 by 56 cm sheets) and chromatographed (ascending) in a solvent consisting of 650 ml of isopropanol, 167 ml of concentrated HCl, and water to 1,000 ml (23). The chromatogram was dried, and the spots were located by ultraviolet light (UV) illumination. The spots were then cut out, minced, and extracted with 5 ml of 0.1 N HCl for 2 h at room temperature (23). Adenine and guanine were quantified by the differential extinction coefficient method (1).

Base analysis by thermal denaturation was performed by the procedure of Mandel and Marmur (12) in 0.1 x SSC. The percentage of G/C was calculated by the formula (21): %G/C = 2.44 (Tm - 81.5 - 16.6 log M), where Tm = thermal denaturation and M = the cation concentration. For 0.1 x SSC, this formula reduced to %G/C = (Tm - 53.1) (2.44). The relationship was verified by using Bacillus subtilis 168 Ind* DNA (the gift of J. E. Urban) and with calf thymus DNA (Sigma Chemical Co.).

**DNA-DNA hybridization.** DNA-DNA hybridizations were performed by the method of Green and Pina (8) and Gillis et al. (7). Trinitrated thymidine-labeled DNA was used as the hybridizing DNA. It was adjusted to a concentration of 1 to 10 µg/ml in 2 x SSC and sheared at 4,000 lb/in in a French pressure cell. Under these conditions, the DNA was reduced to a size of 104 daltons (determined by zonal neutral sucrose sedimentation). Sheared DNA was denatured by placing a flask containing the DNA in a boiling-water bath for 10 min, followed by quick cooling in an ice-water bath (11).

DNA binding experiments were employed in this study (8). Labeled, sheared hybridizing DNA in 1 ml of 2 x SSC plus 10 µlitters of 10% sodium dodecyl sulfate (SDS) was added to each vial (8) and to blank filters. The concentration of hybridizing DNA used varied from 0.5 to 2.5 µg, depending upon the specific activity.

The incubation temperature used for a given experiment was the optimal renaturation temperature (Tm) in 2 x SSC for each DNA, calculated from the formula Tm = (0.51) (%G/C + 47) (7). The Tm values (calculated from Tm data) were 63 C for phage 47 and 77 DNAs, 64 C for phage 80, 81, and 187 DNAs, and 65 C for phage 71 DNA. Incubation was carried out for 20 to 24 h (8).

**RESULTS**

Melting profiles obtained with all the phage genomes were essentially identical, and typical melting profiles of the staphylococcal phage DNAs (phages 47 and 80) and the reference DNAs are shown in Fig. 1. The curves are similar to those reported for other low-molecular-weight double-stranded DNAs (13, 14). The extent of hyperchromicity varied from 38% (for phage 187 DNA) to 46% (for phage 71 DNA),
Table 1. Lytic spectra of typing phage preparations

| Reference propagating strains | Spectra of phage preparations: |
|------------------------------|--------------------------------|
|                              | 47  | 71  | 77  | 80  | 81  | 187 |
|                              | E   | R   | E   | R   | E   | R   | E   | R   | E   | R   |
| 29                           | -   | -   | 0   | -   | -   | 0   | -   | -   | -   | -   |
| 52                           | -   | -   | -   | -   | -   | 1   | -   | 3   | 4   | -   | -   |
| 52A/79                       | -   | -   | -   | -   | -   | -   | -   | 3   | 3   | -   | -   |
| 80                           | -   | -   | -   | -   | -   | 5   | -   | 5   | 5   | -   | -   |
| 3A                           | 0   | 1   | 4   | 4   | 0   | 1   | -   | 1   | -   | 1   | -   |
| 71                           | -   | -   | 5   | 5   | -   | -   | -   | -   | -   | -   | -   |
| 42E                          | -   | -   | -   | -   | -   | -   | -   | 3/0 | 3   | 3   | -   |
| 47                           | 5   | 5   | -   | -   | 5   | 5   | 3   | 3   | -   | 0   | -   |
| 53                           | -   | 0   | -   | -   | 4   | 5   | -   | -   | 1   | -   | -   |
| 54                           | 5   | 5   | -   | -   | 5   | 5   | -   | -   | 3   | 3   | -   |
| 75                           | 0   | 0   | -   | -   | 5   | 5   | 1   | -   | -   | -   | -   |
| 77                           | 0   | 2/0 | -   | -   | 5   | 5   | 0   | 2   | -   | -   | -   |
| 42C                          | 2   | 2   | 4   | 4   | -   | 0   | -   | 0   | 3   | 3   | -   |
| 209                          | -   | -   | -   | -   | -   | -   | -   | 0   | -   | -   | -   |
| 8719                         | -   | -   | 5   | 5   | -   | -   | -   | 0   | -   | -   | -   |
| 187                          | -   | -   | -   | ND  | -   | -   | -   | 5   | 5   | -   | -   |
| 81                           | ND  | 0   | ND  | -   | ND  | 0   | ND  | 5   | 5   | ND  | -   |

*E, Experimental; R, reference data (3, 8); ND, not done; -, negative; 0, inhibition; 2/0, 3/0, reactions which vary from inhibition to lysis (2, 6); 5, ++ reaction with the same dilution that gives ++ on the propagating strain; 4, ++ reaction with dilution $10^4$ to $10^5$ times more concentrated than dilution giving ++ on the propagating strain; 3, ++ reaction with dilution $10^3$ to $10^4$ times more concentrated than dilution giving ++ on the propagating strain; 2, + + reaction with dilution $10^2$ to $10^3$ times more concentrated than dilution giving ++ on the propagating strain; 1, very weak lysis.

and indicated that UV-absorbing material other than double-stranded DNA was not present in significant amounts; contamination would have decreased total hyperchromicity. Furthermore, the DNAs exhibited melting profiles indicative of populations of molecules having single, common mean melting temperatures (12). Bimodal profiles, which might have suggested the presence of more than one DNA species with different melting points, were not observed.

The data from the $T_m$ studies and chemical analyses are summarized in Table 2. The $\%G/C$ values for the staphylococcal phage DNAs ranged from 31% (for serological group A phage 47) to 36% (for serological group B phage 71). A pattern, with respect to serological or lytic groups, was not discernible. The $\%G/C$ values calculated from the $T_m$ data for DNA from the serological group B phages (71 and 80) were higher than the $\%G/C$ values calculated from the $T_m$ data for the other phages, but these differences were not apparent in the results of the chemical analyses (Table 2).

For DNA from phages 47, 81, 80, 77, and 187, the $\%G/C$ values determined from $T_m$ data were within 1% of the values determined by chemical analysis. With phage 71 DNA, however, the $\%G/C$ determined from $T_m$ data was 36%, whereas the value calculated by chemical analysis was 32%. The values are significantly different by the $t$ test ($P < 0.005$), and further
investigation is necessary to establish the meaning of this observation.

The %G/C values reported by Szybalski (24) for the DNAs of phages 80 and 81 agreed with values reported for those phages in this communication (Table 2). In addition, the %G/C values obtained for the reference DNAs, *Bacillus subtilis* 168 Ind+ DNA, and calf thymus DNA agreed closely with published values (Table 2) of their G/C content.

**DNA-DNA hybridization.** At the onset of this investigation, control experiments were performed to define the hybridization system. The results of these experiments are summarized below:

(i) It was found that 12 to 15 h of incubation were sufficient for the hybridization reaction to go to completion. However, for convenience, reactions were routinely incubated for 21 to 24 h.

(ii) Ninety-six percent or more of [H]thymidine-labeled immobilized phage 71 or 81 DNA remained immobilized on membrane filters throughout a 24-h incubation period under incubation conditions employed in this study. These results were unaffected by the presence or absence of sheared, unlabeled (hybridizing) DNA. This is in contrast to the finding of De Ley and Tijtgat (5) that considerable amounts of DNA were released from membrane filters during the incubation period.

(iii) To reduce nonspecific background absorption, the hybridization incubation mixture employed in this study contained 2× SSC plus 0.1% SDS (8). It was found that the effect of 0.1% SDS on the melting of native DNA was essentially negligible. From this finding, it was inferred that the presence of 0.1% SDS in the hybridization reaction mixture would not interfere with the DNA-DNA hybridization reaction.

(iv) The thermal stabilities of hybrids formed under the conditions used in this study were very close to those of native DNA. For example, phage 71 DNA hybrids melted in the temperature range 65 to 70 C in 0.1× SSC. The *Tm* for native phage 71 DNA in the same solvent was 67.8 C (Table 2).

Table 3 shows the percentage of sheared, labeled homologous DNA (relative to the total input counts per minute) bound by the highest concentrations of immobile DNAs used in this investigation. The reported values were obtained under optimal hybridizing conditions (inferred by comparing binding at low and high concentrations of immobile DNAs). The results ranged from 29% homology for phage 80 DNA to 64% homology for DNA from phage 71 and 187.

The reason for the low value with phage 80 DNA is not clear; however, results obtained with phage 80 DNA in the heterologous systems were consistent with results obtained with the other phage DNAs. That is, related DNA sequences hybridized to about the same extent with phage 80 DNA as the control, whereas unrelated

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**Table 2. Determination of percent guanine/cytosine (G/C) contents**

| Source     | Serological group | Lytic group | *Tm* in 0.1× SSC (Tm) | %G/C by *Tm* Individual determinations | %G/C by chemical analysis Individual determinations | Literature values |
|------------|-------------------|-------------|------------------------|---------------------------------------|---------------------------------------------------|------------------|
| Phage 47   | A                 | III         | 65.8                   | 30, 31                                | 33(1), 31(1), 33(1)                                | 32 (32)         |
| Phage 81   | A                 | Miscellaneous | 66.5                   | 32, 33                                | 32(1), 33(1), 31(1), 33(1)                           | 32 (35(29))     |
| Phage 71   | B                 | II          | 67.8                   | 36, 36                                | 28(4), 32(2), 31(2), 34(2), 31(1), 32(1), 33(1)    | 32 (32)         |
| Phage 80   | B                 | I           | 66.8                   | 33, 34                                | 33(1)                                             | 33 (37(29))     |
| Phage 77   | F                 | III         | 66.4                   | 32, 33                                | 32(1), 33(1), 31(1)                                | 32 (32)         |
| Phage 187  | L                 | Miscellaneous | 66.7                   | 33, 33                                | 34(3), 34(2), 34(2)                                | 34 (34(2))      |
| *B. subtilis* 168 Ind+ |                |             | 71.3                   | 44, 45                                | 43(1)                                             | 43 (43(19))     |
| Calf thymus |                 |             | 70.0                   | 41, 42                                | 41(1), 42(1)                                      | 42 (42(19))     |

* Numbers in parentheses indicate number of determinations performed on DNA sample.

* Numbers in parentheses refers to literature references.

* J. E. Urban, Ph.D. thesis,
sequences did not hybridize to any large extent. Table 4 shows the results of the heterologous hybridization reactions. These data were also collected under optimal conditions where all, or almost all, of the sheared DNA (which could bind) was bound to the immobile DNA. In general, reciprocal reactions agreed. There were a few instances where deviations appeared particularly high, such as with phage 80 DNA versus phage 81 DNA (18 and 2%). The reason for this is not clear. It is of interest, however, that differences in reciprocal reactions have been reported in other hybridization systems with phage DNA (4) and in animal viral systems (10). The one instance where a heterologous reaction appeared greater than the homologous reaction (121% for phage 81 hybridizing DNA versus phage 47 immobile DNA) is also not understood.

High degrees of homology (43% or greater) were observed between DNAs from phages of the same serological group. In contrast, much lower degrees of homology (2 to 29%) were observed between DNAs from phages belonging to different serological groups. The lowest homologies were observed between DNAs from phages of serological group A (phages 47 and 81) and serological group B (phages 71 and 80). The serological group L phage 187 appeared to possess more regions of DNA that were homologous with the genomes of serological group B phages than with the genomes of serological group A phages. In contrast, the DNA of serological group F phage 77 showed similar degrees of homology with DNAs from all of the other phages except phage 81. The relatively high degree of homology (29 and 21%) between phages 77 and 47 is of particular interest. These phages belong to different serological groups, yet share the same lytic group (lytic group III). The possibility that phages belonging to particular lytic groups share unique DNA sequences is intriguing and should be investigated further.

**Discussion**

The use of phage typing as a diagnostic tool for the identification and epidemiological characterization of staphylococci has been subject to criticism because of the variability of the phages involved. Although 21 strains (divided according to host range) are routinely used, they are all subject to various restrictions and modifications which could alter host range (16, 17). For example, it has already been established that phage 42D has a hybrid history (19). During the production of the phage 42 family of phages, a lysogen (subsequently called phage 42C) was induced from propagating strain 36. After adaptation of the lysogen to PS 1363, the host-modified variant produced was called 42D. Later, serological methods were used to show that both phages were apparently unrelated to other members of the 42 strain of staphylococcal phages. We attempted, therefore, to investigate some parameters of the genome to establish whether any biochemical rationale existed for the phage groupings as they now exist. Six staphylococcal phages representing all five lytic groups and four serological groups were selected for study of the G/C content of their genome and for relatedness on the basis of hybridization.

It is evident from the data that the base composition of the staphylococcal typing phage

### Table 3. Maximal percent of binding of labeled DNA fragments in homologous hybridization reactions

| Phage DNA | Ratio of sheared DNA/immobile DNA | Maximal binding observed | % |
|-----------|----------------------------------|--------------------------|---|
| 47        | 1:40                             | 64                       |   |
| 81        | 1:40                             | 50                       |   |
| 71        | 1:16                             | 44                       |   |
| 80        | 1:12                             | 29                       |   |
| 77        | 1:20                             | 43                       |   |
| 187       | 1:40                             | 64                       |   |

* Percentage of total labeled (sheared) DNA input.

### Table 4. Heterologous DNA hybridization

| Phage DNA under study | Serological groups of the phages | Lytic groups |
|-----------------------|----------------------------------|--------------|
| 47:81                 | A:A                              | III/Misc     |
| 71:80                 | B:B                              | I/I          |
| 77:47                 | F:A                              | III/III      |
| 77:81                 | F:A                              | III/Misc     |
| 77:71                 | F:B                              | III/II       |
| 77:80                 | F:B                              | III/I        |
| 77:187                | F:L                              | II/Misc      |
| 187:47                | L:A                              | Misc/III     |
| 187:81                | L:A                              | Misc/Misc    |
| 187:71                | L:B                              | Misc/II      |
| 187:80                | L:B                              | Misc/I       |
| 47:71                 | A:B                              | III/III      |
| 47:80                 | A:B                              | III/I        |
| 81:71                 | A:B                              | Misc/II      |
| 81:80                 | A:B                              | Misc/I       |

* Serological groups in same order as phage DNAs.

† In column 1, the first phage DNA listed is sheared DNA; the second phage DNA listed is immobilized DNA. In column 2, the reciprocal reactions are shown.

‡ Miscellaneous.
genomes were similar and little differentiation was possible on the basis of G/C content. No obvious patterns or groupings were apparent with respect to either lytic or serological groupings. The constancy of G/C content among the phages (31 to 36\%) is of interest, however, in that this range encompasses reported G/C values for the host organism, S. aureus: 33\% (24) and 34\% (14).

In contrast to the G/C data, the DNA-DNA hybridization data showed distinct patterns (Table 3). Although it was not possible to segregate these phages on the basis of lytic group, high degrees of homology were found between the genomes of phages in the same serological group. Less homology was observed between the genomes of phages belonging to different serological groups. These findings suggest a positive correlation between serological and genetic relatedness, an observation which was not unexpected. Cowie et al. (4) have shown high degrees of DNA homology among the serologically and genetically related T-even coliphages. Lee and Davidson (11) reported that DNAs from Micrococcus lysodeikticus bacteriophages N\(_1\) and N\(_4\) showed high degrees of homology, and Naylor and Burghi (15) had previously established that phage N\(_4\) was inactivated by antisera to phage N\(_1\). Moreover, DNA from serologically unrelated M. lysodeikticus phage N\(_4\) showed only a low degree of homology with DNA from phage N\(_1\) (11).

The variation encountered in the reciprocal hybridization systems was initially of some concern. It is not unlikely, however, that the homologous sequences were sheared, thus altering the number of stable duplex-forming regions. The result of such shearing action might increase hybridization by causing breaks in long homologous sequences and, consequently, generate a greater number of duplex-forming regions or reduce hybridization by shearing in the center of short homologous regions, resulting in unstable duplexes being formed. Both observations were seen in these data.

The data presented here indicate that, with regard to phage characteristics, the lytic grouping of staphylococcal typing phages represents an arbitrary means of segregation. The serological groupings, on the other hand, can be traced to genome similarities and probably are more reliable. Serological groupings are based primarily on neutralizing antibodies which are specific for the attachment site, whereas lytic groupings are based on the complex interaction of several events and structures. Therefore, the lack of correlation between homologies of lytic and serological groups underscores the complicated nature of the virus-host interaction.

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