Bacteriophages in L Form of *Staphylococcus aureus*†

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Received 4 March 1985/Accepted 24 June 1985

Lysogenicity and phage typability of *Staphylococcus aureus* L-form cells are described. Spontaneously produced phages were found in thin sections of *S. aureus* L colonies. The dimensions of the tail and head resemble those of the morphological group BIII2 (T. Krzywy, I. Durlakowa, A. Kucharewicz-Krukowska, S. Krynski, and S. Slopek, Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. 1 Orig. Reihe A 250:287-295, 1981). Phages 3A and 3C of the international typing set lysed both the bacillary and L form of *S. aureus*.

Tubular and other regular structures have been described in earlier electron microscopic studies of the L forms of various species of bacteria (6, 8, 9, 11). Although some of these structures, occurring in the L form of *Proteus mirabilis*, were described as resembling incomplete phages (phage-like structures) (10, 14), there has until now been no evidence for complete phages in thin-sectioned L-form colonies. In the present paper, physiological and fine structural data are given for a strain of *Staphylococcus aureus* that produced morphologically complete phages during L-form growth.

MATERIALS AND METHODS

The parent strain (*S. aureus* *cla*) was a clinical isolate characterized as *S. aureus* by biochemical data according to the API Staph procedure from Appareils et Procédés d'Identification, Montalieu-Vercieu, France, as well as by positive plasma coagulase and clumping reaction. The L form was induced by fosfomycin as previously reported (20) and maintained on 5% brain heart infusion (Oxoid) with 5% NaCl-1% agar-5% inactivated bovine serum. Unless otherwise stated, 60 µg of fosfomycin per ml was added to this medium.

Phage typing (4) of the parent strain and its L form was carried out with the international *S. aureus* standard set at the German Staphylococcal Phage-Typing Reference Laboratory, University of Bonn.

For fine-structure studies, the L form was subcultured on the above-mentioned agar, but without the antibiotic. Colonies were taken at day 3 of the sixth subculture; no reversion occurred. The colonies were fixed either (i) in osmium tetroxide (16) after 5% NaCl was added to the fixative or (ii) in 2% glutaraldehyde diluted in culture medium without agar, followed by 1% osmium tetroxide in 0.2 M cacodylate buffer. Samples were postfixed in 2% uranyl acetate in water, dehydrated in acetone, and embedded in Epikote 812 (20). Sections were stained with lead citrate (19) and examined with an Elmiskop 101 (Siemens, Berlin, Federal Republic of Germany) at an acceleration voltage of 80 kV.

RESULTS

Some typical biological properties of the parent strain and its L form are given below. From among a large number of *S. aureus* strains transformed into the L form (20), the *cla* mutant, which is described here, produced only small colonies. In contrast to other strains, the faint peripheral zone of

the colonies partially disappeared after more than 3 days of growth.

While the hemolysis and the plasma coagulase activities were retained, the clumping factor was no longer demonstrable in the L form. All three parameters were demonstrable in the parent strain, however. Due to the presence of β -lactamase and to the absence of the cell wall, respectively, parent and L form were resistant to penicillin. The L form was also resistant to oxacillin, whereas the growth of parent bacteria was inhibited.

Lysogenicity. When the fine structure of small L colonies was studied, a spontaneously expressed temperate phage was found in a few cells per colony (Fig. 1), but with a large number of phages per pregnant cell.

Figure 2 shows morphologically complete phages after lysis of the L cells. Under both fixation conditions, i.e., osmium (Fig. 1 and 2) and glutaraldehyde-osmium (Fig. 3 and 4), the heads of mature phages were smaller than empty heads. With reservations discussed later, the approximate dimensions of the prolate head ranged from 43 ± 0.8 by 88 ± 1.2 nm for partially filled or empty heads to 33 ± 0.9 by 84 ± 2 nm for mature heads ($n = 15$). Sections in all possible orientations of empty, partially filled, and completely filled heads are observable in Fig. 3. In this cell, as in the pregnant cell (Fig. 1), there are more than 100 phages per thin section.

The tails clearly exhibited cross-striation (Fig. 3B) and formed very prominent clusters (Fig. 3A) with different orientations. Head production seemed not to be restricted to specific areas within the cytoplasm; phage heads were found at both ends of the bundles of tails that extended from the membrane region to the central region of the cytoplasm (Fig. 3A). In the less compact arrangement found after lysis (Fig. 4A), the long tails (at least 260 nm) looked rather flexible and were found to be assembled even with only partially full heads (Fig. 4A and B). It cannot be decided whether these heads had only been partially filled, representing a physiological feature, or emptied artificially during the manipulations involved in embedding.

Phage typability. After having found that the L-form cells are able to yield spontaneously expressed temperate phages, the multiplication of lytic phages was studied. When 100 times the routine test dilution was administered, phages 3A and 3B of the international standard set, which lysed the parent bacteria, also lysed the L form.

DISCUSSION

Peculiarities in cell morphology are common features in L-form cells. Tubular and nontubular structures as well as

† On the occasion of the 60th birthday of K. G. Lickfeld.

FIG. 1. Part of an L colony of *S. aureus* with one L-form cell containing a high number of phages. Bar, 1 μ m.

FIG. 2. Mature phages with tail and some (larger) tailless empty heads ($\Rightarrow$) in an L colony after burst of the pregnant cell. Bar, 500 nm.

FIG. 3. Panel A, Phage-related particles, which might represent different stages of phage maturation. Empty ($\Rightarrow$), partially filled ($\blacktriangleright$), and filled ($\blacktriangleright$) heads as well as numerous highly ordered phage tails ($\longrightarrow$), both in longitudinal arrangement and cross-sectioned, are found in the cytoplasm of an L-form cell. Bar, 500 nm. Panel B, cross-striation of the long phage tails. Bar, 100 nm.

FIG. 4. Phages in the L colony after lysis of the pregnant cell. The flexible tails (A and C) are connected even with partially filled heads (A and B). Bar, 500 nm (in panel A) and 100 nm (in panels B and C).

paracrystalline inclusion bodies in L-form cells of *Staphylococcus aureus*, *Streptococcus faecalis*, and streptomycetes have been associated with altered protein synthesis (6, 8, 11). Small ellipsoid particles (10) and small dense particles (14), both types found in *P. mirabilis*, were interpreted as products of defective phage synthesis and were thus called phage-like particles, whereas typical phages have so far not been found.

Alterations in adsorption and reproduction of lytic phages in *Bacillus subtilis* L-form cells, in the course of which each step can be defective, have been demonstrated by Jacobson and Landman (12). Depending on the phage strain, the reactions ranged from phages that showed productive lysis to a phage that even failed to adsorb. Besides this dependence on phage particularities, alterations or absence of phage receptors are most likely the reason for the lack of phage typability described for three strains of *S. aureus* (22), in contrast to the lytic action of two typing phages reported in the present study. Also, a transforming activity of free phage DNA has to be considered.

The lysogenicity of the L form (22) and of bacteria reverted from the L form (21) seems to depend on the time elapsed after conversion to the L form, showing a loss of the prophage after prolonged culture.

The results concerning morphology and morphogenesis can only be discussed in respect to phages produced in intact bacteria, since the present micrographs are the first to show different stages of phage maturation in L-form cells of *S. aureus*. Electron microscopical studies, of course, cannot prove that phages are infective and complete (15), but they are reliable and necessary when the production of, e.g., defective phages (2) cannot be proven by lysis of indicator strains.

The differences in phage head dimensions, with empty heads being larger than mature heads, are discussed in connection with DNA encapsidation (for a review, see reference 5). The assembly of phage tails with only partially filled heads does not correspond with the generally accepted scheme of phage maturation (5). It may be a sign of defective phage maturation or of partial DNA degradation of mature phages in vivo or during fixation.

Contrary to intact bacteria (18), no predominant production of phage tails was found adjacent to the cytoplasmic membrane. Presumably due to the more compact cytoplasm of intact bacteria, phage tails are less easily visible (16) than in L-form cells.

Concerning the exact dimensions, the influence of preparation steps (13) has to be taken into account. For example, the mature head of phage λ ranges from 47 nm in thin sections (18) to 54 nm in negative stain (13) to 63 nm in close-to-life cryopreparations (1) to a maximum of 68 nm in X-ray diffraction (7). Being aware of these data and the crucial role of the slicing plane, at least the prolate head and the long tail of the phage found in the *S. aureus* L form are typical of the phage BIII2 morphology group (17). The morphology group is not identical with the lytic group of *S. aureus* phages.

ACKNOWLEDGMENTS

I thank M. Chlupka for continual interest and assistance, W. Lenz (Phage-Typing Reference Laboratory, Bonn) for kindly performing phage typing, and K. G. Lickfeld for constructive comments on the manuscript.

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