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EXPERIMENTAL ARTICLES

The In Vitro Self-Assembly of Supramolecular Structures after the Spontaneous Disassembly of Carotovoricins

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Abstract—The self-assembly of supramolecular structures (empty sheaths and polysheaths of the macromolecular *Erwinia carotovora* bacteriocins) was studied by electron microscopy in the course of 1- to 2-year incubation of phage particles at 4°C. This study showed that the empty sheaths and polysheaths of the bacteriocins of eight *E. carotovora* strains spontaneously assemble at the self-assembly centers (or crystallization centers), which have a diameter of 26–65 nm and contain a dense proteinaceous material. The self-assembly center consists of two components, a primer and the structural protein of contracted sheaths. Empty sheaths assembled in the crystallization centers are polar structures synthesized through the stepwise head-to-tail polymerization of monomeric units. The supramolecular structures of two *E. carotovora* 62A bacteriocins are assembled in a different way. At the early stages of their self-assembly, a reticular structure is formed, which then transforms into very long polysheaths composed of monomers. Along with polysheaths, rounded or lamplike structures 33–117 nm in size composed of the subunits of contracted sheaths are produced. Carotovoricins may serve as suitable objects for the study of the self-assembly of elementary biological structures.

Key words: Erwinia carotovora, macromolecular bacteriocins, self-assembly.

The self-assembly (self-organization) of elementary biological structures is a puzzling phenomenon giving rise to enzymatic assemblies and other biological forms. The problem of self-assembly is part of the general problem of recognition. Self-assembly is based on the universal principle of complementation, which implies an enhancement of the action of superweak physical forces and, in the final analysis, is responsible for the self-organization of biomacromolecules [1].

Some components of bacteriophages, particularly T2 and T4, have already been used for the in vitro study of the self-assembly of elementary supramolecular structures [1–4]. However, the use of simple virion components, such as contracted sheaths of the tail tube, did not allow unambiguous results to be obtained [2–4]. Nevertheless, it is a matter of fact that the empty sheaths of the *Bacillus mycoides* phage 1 are spontaneously synthesized during the long-term incubation of phage particles at low temperatures [4]. When studying the defective temperate bacteriophage particles that are formed in response to the SOS induction of *Erwinia carotovora* cells, I could also observe the formation of analogous structures, i.e., empty sheaths and polysheaths [5], whose number tended to increase in the course of long-term incubation at low temperatures (4–6°C).

The aim of the present work was to study in vitro the self-assembly of supramolecular structures after the spontaneous disassembly of tail-like carotovoricins.

MATERIALS AND METHODS

The seven *Erwinia carotovora* subsp. *carotovora* strains (ECA strains) and two *E. aroideae* strains (EAR strains) used in this study, as well as the methods for obtaining and electron microscopic examination of defective bacteriophages were described in detail earlier [5]. New supramolecular structures were studied by incubating the suspensions of purified carotovoricins containing no less than 1 mg protein/ml ST buffer [5] at 4°C for 1–2 years under axenic conditions. Samples of these suspensions were analyzed under an electron microscope at half-year intervals. The killer activity of the macromolecular *E. carotovora* bacteriocins (carotovoricins) was assayed as described previously [6].

RESULTS

When induced with mitomycin C, *E. carotovora* cells begin to synthesize three types of phage particles: phage heads (PHs), baseplates (BPs), and normal tails (NTs). The sheaths of normal tails can spontaneously transform into contracted sheaths (CSs). In the course

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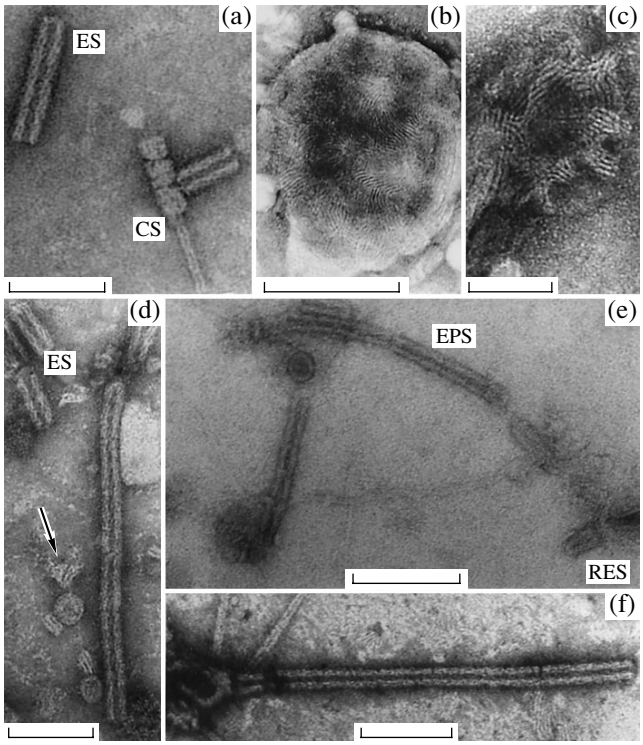

Fig. 1. Empty sheaths and polysheaths of carotovoricins: (a) a partially degraded contracted sheath with the tail tube and a polar empty sheath of TLCA 12-3; aggregated contracted sheaths of the (b) EAR g48 and (c) ECA Ec153 carotovoricins; empty polysheaths of (d) TLCA 29-1, (e) TLCA 25-1, and (f) TLCA 12-3. The arrow points to degraded contracted sheaths without tail tubes (panel d). Scale bars represent 200 nm (panel b) and 100 nm (the other panels). Abbreviations to Figs. 1–4: CS, contracted sheath with the tail tube; ES, empty sheath; EPS, empty polysheath; and RES, rhabdoid empty sheath.

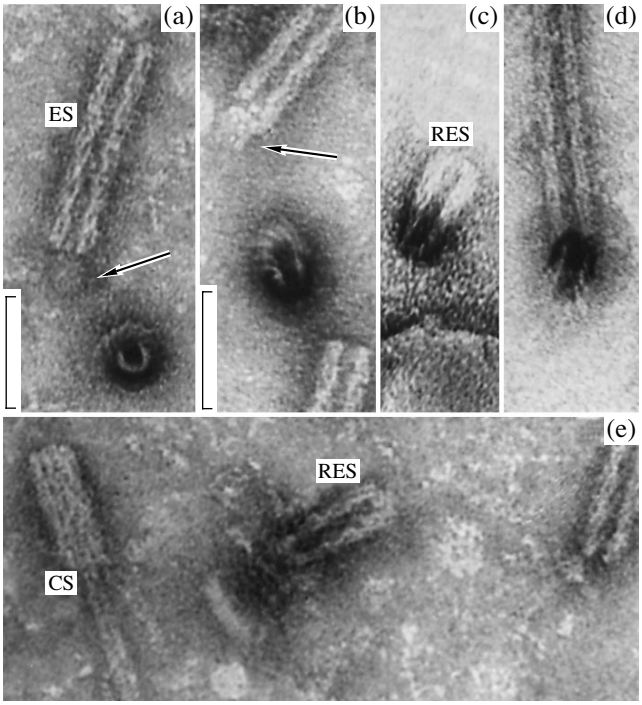

Fig. 2. The formation of (a–c, e) the empty sheath of TLCA 29-2 and (d) the empty polysheath of TLCA 25-1 at the self-assembly centers. The arrows point to spheric particles occurring in the depleted centers (panels a and b). Scale bars represent 50 nm. Magnification for panels a, c–e is the same.

of long-term (1–2 years) storage of CSs at 4°C, they underwent spontaneous destruction of two types. Sometimes, the CSs broke down across the longitudinal axis of the tail, remaining bound to the tail tube (Fig. 1a). Most frequently, however, they underwent swelling and then longitudinal disruption with the formation of planar reticular structures (Figs. 1b–1d). The destruction of CSs destabilized the tail tube, and the latter no longer existed as a macromolecular structure. The planar reticular structures were often observed as large aggregates (Figs. 1b, 1c), from 100 to 300 nm in diameter. Unlike the baseplates of other phages [2, 4], the baseplates of the defective phages of *E. carotovora* were stable and remained intact even after the complete destruction of phage heads and the partial destruction of contracted sheaths.

Empty sheaths (ESs) and empty polysheaths (EPSs) of the tail-like carotovoricins (TLCAs) of the ECA strains were formed simultaneously with the destruction of contracted sheaths. In the course of 2-year storage, the carotovoricins of the ECA strains 35A, Ec153, 48A, 59A, J2, and 4A and of the EAR strains g48 and 3A produced ESs and EPSs of different lengths (Figs. 1a, 1d, 1e), whose number increased with time.

The average width of empty sheaths, estimated through the linear measurements of no less than 30 structures, was equal, within the measurement error, to the width of the contracted sheaths of the respective TLCAs (see [5] and table). The length of the empty sheaths was variable (the standard deviation was no less than 10%) and, as a rule, differed from the length of the contracted sheaths. The EPSs were several times longer than the contracted sheaths. The length ratios of the empty and contracted sheaths were not integers in virtually all cases, suggesting that these structures are of different origin.

As can be seen from Figs. 1d–1f, all empty polysheaths, which were 2 to 8 times longer than the contracted sheaths, were divided into segments by thin septa. It can be suggested that EPSs resulted from the head-to-tail polymerization of the contracted sheaths detached from the tail tube. If this is the case, the monomeric units of a polysheath must be identical and their length must correspond to the length of the contracted sheaths that have been polymerized. However, observations showed that the monomeric units have different lengths. This is clearly seen from the analysis of two

The sizes of the contracted sheaths of tail tubes and the empty sheaths and polysheaths of tail-like carotovoricins

Strain	Carotovoricin	Contracted sheath, nm		Empty sheath or polysheath, nm		Length ratio*
		L	*W*	*L*	*W*	
ECA 35A	TLCA 12-1	61.0	21.8	113–463	21.5	1.85–7.60
	TLCA 12-2	62.9	23.4	187–265	23.4	2.97–4.21
	TLCA 12-3	60.1	25.4	190	25.0	3.16
ECA J2	TLCA 44-2	49.4	17.0	48.3	17.4	0.98
	TLCA 44-3	68.4	20.2	–	–	–
ECA Ec153	TLCA 29-1	54.4	18.3	54.6	18.1	1.00
	TLCA 29-2	57.3	21.0	55.7	20.7	0.97
ECA 48A	TLCA 25-1	68.5	21.1	61.2	21.3	0.89
	TLCA 25-2	66.8	26.8	–	–	–
EAR 3A	TLCA 11-1	70.0	26.0	–	–	–
	TLCA 11-2	68.5	28.0	60.3	28.0	0.88
EAR g48	TLCA 2-1	58.3	21.5	41.4	20.7	0.71
	TLCA 2-2	61.6	25.4	–	–	–
ECA 59A	TLCA 55-1	55.3	21.8	64.9	22.2	1.17
	TLCA 55-2	61.4	17.2	54.9	18.4	0.89
ECA 4A	TLCA 36-1	66.4	24.7	60.2	24.7	0.90
	TLCA 36-2	68.0	27.3	–	–	–

Note: "–" indicates that the respective particles were not observed.
* Length ratio is the ratio of the length of an empty sheath (or polysheath) to the whole length of the corresponding contracted sheath.
L is length, and *W* is width.

polysheaths of TLCA 25-1 presented in Fig. 1e, whose monomeric units are 18.8, 37.7, 43.9, 47.0, and 56.5 nm long. For comparison, the length of the respective contracted sheath was 68.5 nm (see table).

Analysis of the electron images of the empty sheaths and polysheaths of various carotovoricins showed that, in contrast to contracted sheaths, they are polar structures (Figs. 1a, 1d, 1e). One of their ends was typically rounded and closed (this end may be called "a head"), whereas the other was open and had the same diameter as the main portion of the empty sheath (this end may be called "a tail"). Many ESs were rhabdoid in shape and shorter than the usual empty sheaths (Fig. 1e). As a rule, rhabdoid empty sheaths (RESs) were localized close to the polysheath "head" (Figs. 1d and 1e).

In an attempt to reveal the role of these structures in self-assembly, the suspension of the ECA Ec153 carotovoricins stored for one year was thoroughly examined under the electron microscope. The examination showed that there are two types of RESs, which corresponded to the empty sheaths of TLCA 29-1 and TLCA 29-2 (see table). Both RES types were 38–52 nm long, i.e., shorter than ESs. The tail portions of TLCA 29-1 and TLCA 29-2 were, respectively, 11 and 8% wider than the empty sheaths of these carotovoricins. The analysis of the rhabdoid sheaths of the TLCA 25-1 of strain ECA 48A gave similar results. The RESs of this carotovoricin had a length of 38–50 nm, the width of the tail portions being 24 nm (11% wider than in the respective empty sheaths (see table)).

These data suggest that rhabdoid empty sheaths may be intermediates of the self-assembly of empty sheaths. This suggestion was confirmed by the discovery of self-assembly (crystallization) centers for the rhabdoid sheaths of TLCA 29-1 and TLCA 29-2. As a rule, these peculiar structures looked like regular circles with a mean diameter of 26 nm. At the circle center, there was a thin semicircle about 10 nm in diameter. In the circle region opposite to the semicircle, there was a thickened structure resembling an empty sheath (Fig. 2a). In a three-dimensional space, these structures must obviously represent two opposite hemispheres, the smaller being enclosed into the larger one. The detailed analysis of these supramolecular structures and the rhabdoid empty sheaths associated with the crystallization centers (Figs. 2b and 2c) allowed the following hypothetical model for the self-assembly of the empty sheaths of TLCA 29-1 and TLCA 29-2 to be proposed:

The self-assembly of empty sheaths begins at the self-assembly (crystallization) centers, which contain a dense proteinaceous material. The interaction between the protein components of the regular spherical crystallization centers must be very strong, since these centers remain unchanged even when the self-assembly of the

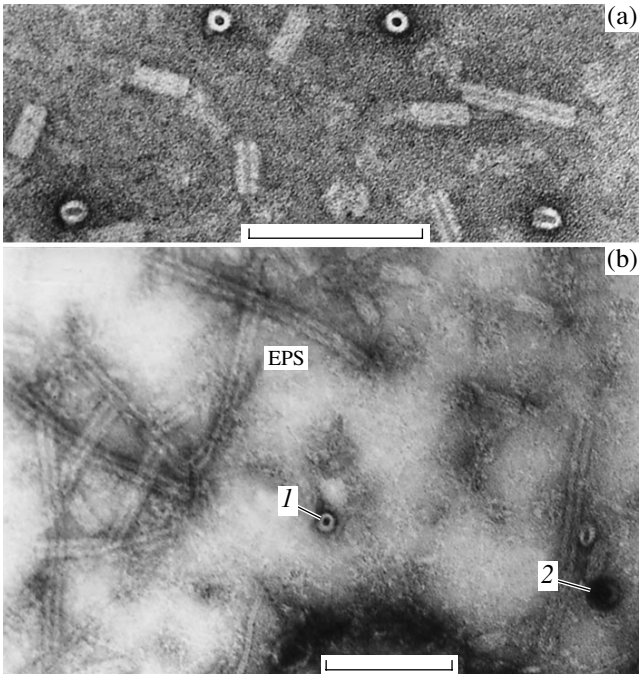

Fig. 3. The crystallization centers of the empty sheaths and polysheaths of (a) TLCA 11-2 and (b) TLCA 36-1. The arrows point to two different centers, *1* and *2*, of TLCA 36-1. The scale bars represent 200 nm.

empty sheaths is completed (Fig. 2a). The hemispheric supramolecular structures involved in the self-assembly presumably contain two different proteins (or preassembly protein components). The second stage of the self-assembly lies in the growth of the tail portions of rhabdoid empty sheaths (Fig. 2b), during which the smaller hemisphere undergoes structural alterations and binds to the RES head. The density of the crystallization centers decreases, particularly on their periphery, and their shape becomes close to a prolate ellipsoid of revolution 36×45 nm in size (Fig. 2b). The described changes in the spatial organization of the self-assembly centers may be due to the release of the synthesized structures into the surrounding medium, since the larger axis of the spheroid is oriented along the longitudinal axis of the RESs. At the third stage, the synthesized structure becomes a rhabdoid empty sheath, whose tail portion appears outside the crystallization center (Fig. 2c). Empty sheaths are likely finished when they separate from the crystallization centers (Figs. 2b, 2c, 2e). In this case, the width of their tail portions decreases, whereas the packing density of the structural subunits increases to become close to that of contracted sheaths (Fig. 2e). Some synthesized structures remain bound to the crystallization centers. The aggregates of these structures have a low density and each possesses inside a spheric structure 12–14 nm in diameter (Figs. 2a and 2b). The latter structures are likely related to the smaller hemispheres of the self-assembly centers and are composed of the same protein. Both described supramolecular structures are obviously involved in the self-assembly of the empty sheaths of the ECA Ec153 carotovoricins as primers, judging from the fact that the amount of these structures is considerably lower than the amount of the structural proteins of the empty sheaths. They may also be involved in the formation of polar empty sheaths and polysheaths through binding to the "heads" of rhabdoid sheaths (see above). The primers may be composed of the proteins of tail tubes, which, as mentioned above, are degraded prior to the partial spontaneous destruction of the contracted sheaths of carotovoricins.

Further studies showed that the stored suspensions of the carotovoricins of strains EAR 3A and ECA 4A also contained crystallization centers (Fig. 3) similar to those of TLCA 29-1 and TLCA 29-2. The suspension of TLCA 11-2 stored for one year contained, in addition to contracted sheaths, empty sheaths, and small polysheaths (Fig. 3a), up to 20% of 48.5-nm crystallization centers, in which rounded and rhabdoid developing ESs could be observed. At the same time, these carotovoricin suspensions did not show the presence of small semicircular structures analogous to those of the ECA Ec153 carotovoricins.

The TLCA 36-1 of strain ECA 4A was characterized by the presence of two types of self-assembly centers (Fig. 3b). The self-assembly centers of the first type were 29.5 nm in diameter and had 23.6-nm rings inside. The self-assembly centers of the second type were larger (50–60 nm in diameter), and some of them contained a small hemisphere 15.7 nm in size. Similar centers, from 53.2 to 65.7 nm in diameter, were also found in the suspension of TLCA 25-1 of strain ECA 48A stored for one year.

An analysis showed that the empty polysheaths of carotovoricins may be formed from polar empty sheaths through their head-to-tail polymerization. Alternatively, EPSa may be formed in the crystallization centers simultaneously with ESs. Further studies of the self-assembly centers and the EPSs of TLCA 36-1 (Fig. 3b) showed that the number of second-type centers (as mentioned above, they are 20% more variable in diameter and 1.7 to 2 times larger than the first-type centers) exceeded the number of the latter by 60–80%. The occurrence frequency of long empty polysheaths decreased exponentially with their length and correlated with the occurrence frequency of the large self-assembly centers. These data suggest that EPSs may be assembled at the large centers (50–60 nm), whereas the ESs of TLCA 36-1 may be assembled at the small centers (29.5 nm).

Indirect confirmation of this suggestion came from the analysis of the occurrence rate of the aggregates of contracted sheaths formed immediately after their destruction (Figs. 1b and 1c). The percentage of large spheric aggregates with a mean diameter of 312 nm (Fig. 1b) formed in the suspensions of the EAR g48

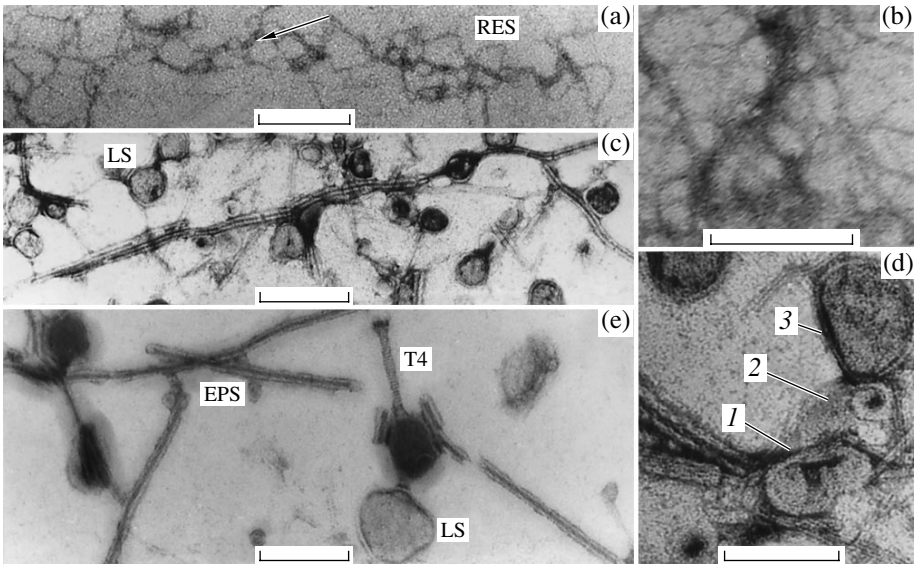

Fig. 4. The in vitro self-assembly of the empty polysheaths and lamplike structures (LSs) of TLCA 5-1 and TLCA 5-2: (a and b), reticular structure; (c–d), long empty polysheaths and LSs. The arrows point to (*1*) an ellipsoid particle, (*2*) a rounded particle, and (*3*) a sheathed particle. T4 is the *Escherichia coli* T4D phage. The scale bars represent 200 nm (panel c) and 100 nm (the other panels).

carotovoricins was as low as 17%, whereas the other 87% of aggregates have a diameter of 127 nm. These data suggest that centers for the self-assembly of empty sheaths and polysheaths are formed from different spherical aggregates of the material of dissolved contracted sheaths. For this reason, the resultant self-assembly centers have a different size and density (Fig. 3b).

Direct evidence for the formation of empty polysheaths at the self-assembly centers came from the detailed electron microscopic investigation of the suspension of strain ECA 48A carotovoricins stored for one year. As can be seen from Fig. 2d, the polysheath of TLCA 25-1 associated with the large 65-nm self-assembly center was formed as the result of penetration of the "head" of a rhabdoid empty sheath situated at the self-assembly center into the open tail portion of a partially synthesized polysheath. In the course of polymerization, the polysheath moves along the radius of the center and then releases into the medium. Unlike the self-assembly of ESs, the self-assembly of empty polysheaths is a complex and presumably stepwise process.

Noteworthy is the self-assembly of empty polysheaths after the destruction of the TLCA 5-1 and TLCA 5-2 of strain ECA 62A. The phage particles of the type of heads, baseplates, and tail tubes of this strain were found to be less stable than the analogous particles of the other strains studied. The storage of a suspension of the carotovoricins of this strain at 4°C for 5–7 months led to their complete inactivation, whereas the killer activity of the carotovoricins of the other strains after such a storage fell insignificantly, if at all. The inactivation of TLCA 5-1 and TLCA 5-2 was associated with the loss of opalescence, typical of suspensions of phage particles, as well as with a spontaneous precipitation of the suspended material onto the test tube bottom. After 5–6 months of storage, the biologically inactive aggregated suspensions of carotovoricins contained no structures of the type of phage heads, baseplates, normal tails, and contracted sheaths, but did contain a small number of naked tail tubes without contracted sheaths and a number of long filamentous structures resembling a cross-linked felt network with very thin filaments on the periphery (Fig. 4a). The central regions of these reticular structures had thickened formations 19 to 25 nm in width, some of which were sigmoid (Fig. 4b) and resembled empty polysheaths. Six months after the reticular structures began to form, very long empty polysheaths could be observed (Fig. 4c). These polysheaths were obviously related to the earlier formed reticular structures presented in Fig. 4a. The EPSs of TLCA 5-1 and TLCA 5-2, like the EPSs of the other carotovoricins, were composed of particular monomeric units, i.e., septate empty sheaths (Fig. 4d). As can be seen from this figure, the weak action of a T4 phage particle on a polysheath is sufficient to break it into particular empty sheaths. With respect to the length and width of the component monomers, empty polysheaths can be divided into two groups, which correspond to the two types of the parent contracted sheaths of TLCA 5-1 and TLCA 5-2 (15 and 17 nm, respectively). None of the observed polysheaths included the ES monomers of both carotovoricins.

The stored suspensions of the ECA 62A carotovoricins also contained lamplike structures (LSs) with flexible sheaths from 33 to 117 nm in diameter (Figs. 4c–4e).

About 20% of the LSs were small (38 nm in diameter), rounded, and had a dark central part (Fig. 4e). About 25% of the LSs had a diameter of 52.2 nm and a different structure. Some of them had a distinct sheath 4–5 nm thick. The LSs lacking sheaths were often ellipsoid or had the shape of a cabbage leaf (Fig. 4e). Some particles of this size resembled the ghosts of phage heads. The remaining 55% of the LSs had a diameter of 62 to 117 nm (Figs. 4c–4e). Further analysis showed that the LSs are not related to intact phage heads (Fig. 4e) or the phage particle ghosts typical of the ECA 62A carotovoricins [5]. The thickness of the LS sheaths (about 5 nm) was close to that of the empty sheaths and polysheaths of all of the carotovoricins studied and the long polysheaths of the parent ECA 62A strain.

A comparative analysis of the contracted sheaths of tail tubes and the empty polysheaths of TLCA 5-1 and TLCA 5-2 and ellipsoid particles showed that all of them are similarly organized and probably composed of similar protein subunits (Fig. 4e). The lamplike structures and long empty polysheaths may be the products of the self-assembly of the material resulting from the destruction of the contracted sheaths of TLCA 5-1 and TLCA 5-2.

DISCUSSION

The self-assembly of empty sheaths and polysheaths in vitro after the disassembly of the contracted sheaths of the tail tubes of the defective phages (macromolecular bacteriocins) of *E. carotovora*, including the very long polysheaths and the lamplike structures of the ECA 62A carotovoricins, may represent a low-order morphopoesis [2], although some authors believe that the self-assembly of phage particles is a higher-order process involving not only structural proteins but also other agents [7]. According to my observations, the self-assembly of empty sheaths and polysheaths is preceded by the disassembly of tail tubes and their contracted sheaths (Figs. 1b–1d). The latter process occurs most likely in large spheric structures (Figs. 1b and 1c), which result from the side-to-side aggregation of contracted sheaths or from the complementary interaction [1] of the products of their partial destruction along the longitudinal axis.

The self-assembly (crystallization) centers of the empty sheaths and polysheaths of carotovoricins (Figs. 2 and 3), which are formed from the spheric aggregates of material resulted from the degradation of contracted sheaths and, probably, some tail tubes, have not yet been described in the literature. Empty sheaths and polysheaths are assembled only at the crystallization centers, which consist of two components, a primer and the structural protein of contracted sheaths. In well studied systems, either protein molecules or nucleic acids (in particular, RNA [8]), or the degradation products of phage particles [1] and other structures [9] may serve as a primer. The origin of the primer involved in the self-assembly of the empty sheaths of carotovoricins remains unknown.

Empty polysheaths are assembled at the crystallization centers (Fig. 2) most likely through the "fish tail" polymerization, similarly to the self-assembly of bacterial flagella [9]. A minor difference lies in that the self-assembly of empty sheaths is a stepwise process giving rise to polar empty polysheaths composed of monomeric units (Figs. 1d–1f). The self-assembly of empty sheaths and polysheaths is carotovoricin-specific (Table 1).

The supramolecular structures of two *E. carotovora* 62A bacteriocins are assembled in a different way, i.e., through the formation of long reticular structures (Figs. 4a and 4b). The formation of very long empty polysheaths and the lamplike structures from the structural proteins of the contracted sheaths of TLCA 5-1 and TLCA 5-2 have not yet been described in the literature. The self-assembly of LSs is a complex process, which cannot be studied in detail solely by electron microscopic methods.

The self-assembly of the empty sheaths and polysheaths of carotovoricins can serve as a suitable model for the study of the self-assembly of elementary biological structures. The study of the self-assembly centers may provide information on the strength of intermolecular interactions and on other thermodynamic parameters of the self-assembly process.

The biological role of the empty sheaths, empty polysheath, and the lamplike structures of carotovoricins is unknown and requires further investigations.

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