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EXPERIMENTAL ARTICLES

Analysis of the Morphological Variants Arising During S⟷R Dissociation in *Bacillus thuringiensis*

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Abstract—Physiological, biochemical, and serologic characteristics of 24 clones of R variants spontaneously emerging from the wild type strain of *Bacillus thuringiensis* biotype *dendrolimus* and of 25 clones of S revertants resulting from the plating of one of the R variants are investigated. It is shown that the efficiency of spore formation is the only characteristic among those investigated which correlated with colony morphology of the S or R type. All R variants were oligo- or asporogenous; they usually differed in characteristics of adaptive importance. The process of spore formation was restored in the S revertants, but they differed both from the wild type and from each other. In contrast to the initial forms, R variants and S revertants most commonly exhibited the capability to ferment sucrose. Based on the data obtained, an assumption was made concerning the nature of dissociation in the strain under investigation.

Key words: Bacillus thuringiensis, S⟷R dissociation.

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Bacillus thuringiensis is a well-known species of entomopathogenic bacilli which has been used as a biological insecticide for more than thirty years. The main distinctive feature of this species is the formation of a protein crystal of δ endotoxin during sporulation. Recognition of the leading role of this toxin in insect death has focused the attention of many researchers on this feature and led to detailed understanding of its molecular biology and genetic control, and the mechanism of its effect on insect larvae [1]. Less attention has been paid to the phenomenon known as "phenotypic dissociation," long-known for *B. thuringiensis*. Dissociation is typically recorded visually, as in the initial clone culture this process results in emergence of cells forming colonies with morphology distinct from that of the initial clone (S or R) with the frequency substantially exceeding the frequency of natural mutations. The characteristics of the clone with modified morphology are inherited, but reversion to the initial phenotype is possible with high frequency [2]. Investigation of this phenomenon in various subspecies of *B. thuringiensis* revealed that the emerging morphological variants often differed from the initial forms in the disrupted processes of spore and crystal formation [3, 4]. Furthermore, it was established earlier that asporogenous and oligospore-forming variants exhibit modifications in the biochemical composition and structure of cell walls, motility, nature of chemical response, metabolic activity, growth characteristics on complete and synthetic media, antibiotic and phage resistance, and UV-, osmo-, and temperature sensitivity [3–9]. Unfortunately, in all these studies, the properties of morphological variants obtained from the single colony were generally compared with the initial phenotype. In our opinion, this does not provide a complete picture of the dissociation process or understanding of its biological meaning and mechanisms, which remain obscure.

The goal of the present work was to determine which phenotypic changes are possible in individual clones of one of *B. thuringiensis* strains as the result of dissociation. For this purpose, a number of characteristics were investigated for variations in colony morphology, which emerged spontaneously or due to UV irradiation in the plating of one of the S clones of an initially spore- and crystal-forming strain of the wild type. Then, similar features were analyzed in the revertants that spontaneously emerged from the clone culture of one of the colony morphology variants which lacked both spores and toxin crystals. The data obtained allow us to conclude that changes in the colony morphology of the analyzed strain are the result of the disruption of the process of spore formation not connected with a loss of genetic material. An assumption was made about the possible mechanisms of the preservation of the diagnostically significant characteristics and of the widening of the adaptive capabilities of bacteria in the course of dissociation of their populations.

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MATERIALS AND METHODS

In this work, the type strain of *Bacillus thuringiensis* 49, serotype H_{4a4b}, serovar *sotto*/*dendrolimus*, biotype *dendrolimus* was used; the strain was obtained from the collection of the Museum of Microbiology of Irkutsk State University. This is the basis of the strain stored in BGSC (no. 4E2, code HD7). Bacteria cultivation and analysis of colony morphology were performed on solid LB (Luria–Bertani) nutrient medium at 28°C. The same medium without agar was used as the liquid medium. To reveal the requirement of growth factors, the Nickerson and Bulla synthetic minimum medium (N) was used, supplemented with solutions of *L*-amino acids up to the final concentration 20 μg/ml of medium.

The response to antibiotics was determined on LB medium supplemented with antibiotic solutions (50 μg/ml). The cell suspension was plated on selective media by means of a 26-pin replicator. The growth was assayed on the first, third, and seventh days of bacterial cultivation. The physiological and biochemical characteristics of the variants and the initial strain were determined according to the generally accepted procedures [11].

Analysis of the antigenic characteristics was performed by a lamellar (spot) agglutination reaction using one of the dilutions of the immune monoreceptor diagnostic O serum (1 : 50 or 1 : 100) with the titer of antibodies 1 : 3200. The thermostable cellular O antigen was obtained by heating the suspensions of one-day culture cells for 2 h in a boiling water bath [12].

The presence of heat-resistant spores was quantitatively assayed by colony (CFU) counts after inoculation of LB medium with the spore suspension (ten-day culture) before and after heating for 30 min at 75°C. The ratio of spores, crystals, and vegetative cells was analyzed in 7–10 day cultures on LB medium by the Brid method. The preparations were made from the suspension with bacteria concentration 10^6 ml^{-1}, stained with Comassie brilliant blue [13], and analyzed under a Jenamed Carl Zeiss Jena microscope in a 100×1.25 oil immersion system. The counts were performed in 30 fields of vision; the ratio of spores and crystals of toxin was assayed by the χ^2 method.

RESULTS

The cells of the initial strain *B. thuringiensis* 49 on solid media formed colonies of S type with smooth bright surface, white color, and slurred consistency. On the first day of cultivation, the R variant colonies which emerged spontaneously or under UV illumination did not morphologically differ from the colonies of the initial S variant. However, as the cells aged, the surface of the R colonies became plain and fine-grained, the color turned to grey, and the consistency became slightly dry. Initially, 25 independently formed colonies of the R variant were isolated. Seven clones emerged spontaneously (R1–R3, R10, and R23–R25); one variant (R11) arose after radiation treatment of the six-h culture with a dose of UV light which resulted in $71.4 \pm 24.90\%$ cell survival (UVL-71); five variants (R4, R5, R12, R13, and R21) were isolated after treatment with a dose of UV light which resulted in the survival of $22.4 \pm 5.80\%$ of the cells (UVL-22); and 12 variants (R6–R9, R14–R21, and R22) were obtained in experiments with the survival level of $6.5 \pm 3.98\%$ after radiation treatment (UVL-7). The cells of one variant (R5) proved unviable in the subsequent passages. All viable cells were analyzed for the characteristics used to differentiate between *B. thuringiensis* subspecies: character of growth in liquid medium; formation of acetylmethylcarbinol (AMC), urease, pigment, spores, and toxin crystals; capability to hydrolyze sucrose, mannose, salicin, and esculin; and presence of proteolytic, lecithinase, and amylolytic activities. Furthermore, since the initial strain required valine and leucine, the amino acid requirements of the variants were also analyzed. Cell suspensions of one-day cultures were transferred to a number of media; each of them lacked only one amino acid. Growth on the minimum media containing all amino acids and on the same medium without amino acids served as controls. In view of the data concerning the changes in the cell wall [7] of the variants of colony morphology, their response to the presence of antibiotics of the penicillin group was investigated.

The analysis revealed that most of the main diagnostically important characteristics did not change during the S⟶R dissociation. No differences were revealed between the analyzed clones of R variants and the initial S form in their growth in liquid medium; agglutination reaction with the diagnostic O sera to the initial strain 49; formation of acetylmethylcarbinol, pigment, and urease; or hydrolysis of esculin, salicin, and mannose. All the morphological variants had proteolytic, amylolytic, and lecithinase activities; quantitatively, their levels could change slightly either to larger or to smaller values. Differences in the size of colonies formed on LB medium after 27 h of cultivation at 28°C were revealed. The average colony diameter in various clones of R variants (0.19 ± 0.006 cm) was reliably ($P < 0.05$) higher then that of the initial strain (0.16 ± 0.005 cm). Significant differences were also found in the capability to ferment sucrose, in the level of proteolytic activity, in growth on selective media with amino acids or antibiotics, and in the effectiveness of sporulation and toxin formation. The characteristics of several clones of R variants are presented in Table 1. Only the characteristics different from the initial S form are shown.

We did not find variants completely identical in all characteristics; each of them possessed individual peculiarities. No correlation in the character of the changes was found. Neither was any effect revealed of the conditions of variant isolation (spontaneous or due to UV light). In contrast to the initial strain 49, 50% of the investigated clones of R variants were capable of fermenting sucrose. Some variants had a decreased pro-

Table 1. Characteristics of the R variants obtained from *B. thuringiensis* strain 49

Strain and its origin	CFU/ml		Ratio		Hydrolysis of sucrose	Proteolytic activity	Resistance to antibiotics*	Growth delay in the first day on N medium without amino acid**
	before heating	after heating	spores : crystals	χ^2 1 : 1				
49, wild type	4.2×10^8	6.7×10^8	1 : 1	1.70	–	+	A, C, M, P	V; L
R2, spontaneously	2.4×10^8	1.4×10^2	1 : 1	3.32	+	+	A	V; L; A; D; H; I
R3, spontaneously	1.8×10^8	1.0×10^2	1 : 1	2.43	+	+/–	A, C, M, P	V; L; H
R10, spontaneously	0.8×10^8	0.3×10^1	1 : 1	0.02	–	+	–	V; L; H
R23, spontaneously	0.5×10^8	0	0 : 0	0	–	+/–	–	V; L; A; E; H; I; M; T
R11, UVL-71	1.5×10^8	0	0 : 0	0	–	+	–	V; L
R13, UVL-22	4.0×10^8	3.2×10^7	1.5 : 1	10.79	+	+	–	V; L; C; E; H
R6. UVL-7	1.1×10^8	0.1×10^2	1 : 1	0.01	+	+	A. C. M, P	V; L; H
R8, UVL-7	2.6×10^8	1.6×10^3	1 : 1	0.37	–	+	A, C, M, P	V; L
R14, UVL-7	2.9×10^8	1.9×10^3	1 : 1	0.02	–	+	A, C, M, P	V; L
R15, UVL-7	3.1×10^8	2.1×10^3	1 : 1	0.46	–	+	A, C, M, P	V; L; D; H; K; T
R19, UVL-7	2.8×10^8	1.7×10^3	1 : 1	0.07	–	+/–	A, C, M	V; L; D; G; H; I; K; R; S; T
R22, UVL-7	1.3×10^8	0.1×10^2	1 : 1	0.11	+	+	A, C	V; L

Notes: * Antibiotics: P, penicillin; A, ampicillin; C, carbenicillin; M, methicillin.

** Amino acids: alanine, A; arginine, R; aspartic acid, D; histidine, H; valine, V; glycine, G; glutamic acid, E; isoleucine, I; leucine, L; lysine, K, methionine, M, serine, S; threonine, T; cysteine, C; "+," the presence of activity; "–," the absence of activity or resistance; "[+/–]," weak activity.

teolytic activity. Five variants were sensitive to all the antibiotics applied; however, half of the investigated clones did not change on media with antibiotics. Most of the variants varied in the character of growth on selective media during the first 48 h of cultivation. All the R variants and the wild S type grew normally on the medium supplemented with all 20 amino acids; after 24 h of cultivation, their growth character did not differ from the growth on complete LB medium. The clones of the wild strain did not grow on media with all amino acids except valine and leucine. 24-h growth delay was stably observed on the medium without methionine. Growth delay or weak growth was observed after 24 h on the medium without threonine. Only six of the variants were similar to the initial strain in this characteristic. The others were characterized by a delay of growth on the media without other amino acids (Table 1), except for the aromatic ones. Growth delay on the medium without histidine was observed for 75% of the examined variants; for 55% on the medium without isoleucine; for 45% on the medium without glycine; for 35% on the media without aspartic acid or threonine. Growth was observed after four days of incubation on all selective media lacking one amino acid. The common difference of the R variants was the total absence of growth on mineral medium without amino acids in contrast to the weak growth of the wild type strain on the fifth–seventh day.

The single characteristic common to all the emerging morphological variants was disrupted spore formation. None of the examined clones of R variants formed spores with the same efficiency as the initial strain. The morphological variants R8, R11, and R14 differed from the initial strain only in this characteristic. Microscopy revealed that the wild type strain 49 formed prospores on the solid LB medium on the second day of cultivation; on the fifth day, free spores and toxin crystals were observed, while nonspore-forming cells were very rare. In none of the R variant clones were spores and crystals revealed under the same conditions. Only on the seventh–tenth day was the formation of spores and toxin crystals observed along with vegetative cells; their quantity varied for different variants. This finding was confirmed by the results of the estimation of heat-resistant spores (Table 1). In the preparations of the R13, 47 nonsporulating cells were found (348 cells counted); in the R10 variant, 601 cells of 654 cells were of this type. In the preparations of the R23 variant, no spores were found, and inoculation of the heated cell suspension with initial cell concentration of 0.5×10^8 CFU/ml did

not reveal any colonies. In most cases, the decrease in spore formation by the variants correlated with decreased effectiveness of crystal formation. Only in the R13 variant was the spore number reliably 1.5-fold higher than the crystal number (χ^2 fact. 10.8). Various clones of R variants also differed in cell morphology: some variants had elongated cells in chains, weakly stained, and had destructured or structured cytoplasm; other variants had single cells of coiled or even toroidal shape.

No correlation was found between the degree of disturbances of spore formation and changes in other characteristics. Thus, the R6 variant, with low effectiveness of spore formation, was resistant to all antibiotics, and the R13 variant, with the least degree of disturbance of spore formation, was sensitive to all antibiotics of the penicillin group.

The data obtained revealed that variants with inheritable individual changes emerged from strain 49 in the process of S⟶R dissociation. These changes do not affect the basic metabolic processes which determine the species characteristics (except for spore formation). Apparently, as has been noted by other researches [3], the disturbances of spore formation are related to the changes of colony morphology and allow us to register the variability of *B. thuringiensis* cultures.

To investigate the possibility and characteristics of reverse R⟶S transition, the spontaneously emerging variant R23 was chosen; it differed from the initial strain 49 in the total lack of spore and crystal formation, had impaired proteolytic activity, was sensitive to all antibiotics, and exhibited a growth delay on eight selective media (Table 1). The experiments with UV irradiation revealed that the cells of this variant were more sensitive to UV light. After treatment with a dose which enabled the survival of $22.4 \pm 5.80\%$ of the cells of the initial strain, only $5.0 \pm 2.96\%$ of the cells of the R23 variant survived. However, S revertants were obtained from this strain. Eleven clones (S1–S11) arose spontaneously during the usual plating of the R23 variant on LB medium. Other clones (S12–S25) were isolated after plating of a six-h culture of the R23 variant irradiated with the UV dose which provided the cell survival of $87.9 \pm 5.64\%$.

Analysis of the physiological, biochemical, and serological characteristics of S revertants did not reveal changes in the features common to the asporogenous variant R23 and the initial strain 49. All of them did not form a film during static growth in liquid medium, exhibited positive reaction of agglutination with the diagnostic O sera to the initial strain 49, did not form pigment, urease, did not hydrolyze salicin and mannose, formed acetylmethylcarbinol, hydrolyzed esculin, and had amylolytic and lecithinase activities.

In contrast to the R23 variant, all the S revertants obtained from it were characterized by levels of proteolytic activity, resistance to antibiotics of the penicillin group, and effectiveness of spore and crystal formation restored to the level of the initial wild type strain (Table 2). Only one of the revertants exhibited significant growth delay on selective media, thus, in general, retaining this feature of the R23 variant. Other clones did not differ in this respect from strain 49, expect that several S revertants had more prolonged growth delays on the medium without methionine and/or threonine than the wild type strain. As can be seen from Table 2, the greatest instability among R⟶S dissociants was again in the capability to ferment sucrose. Both spontaneous S revertants (90.9%) and the S clones induced by UV irradiation (69.2%) obtained the capacity to hydrolyze sucrose in contrast to both the R23 variant and the initial strain 49.

In spite of the overall picture of reversion to the wild type phenotype, intent analysis of a combination of properties for each variant revealed neither exact conformity to it, nor complete similarity between revertants. Thus, among the ten revertants sharing with the wild type strain the growth delay on media without valine or leucine, eight revertants fermented sucrose and two did not. Among the eight sucrose-fermenting revertants, clones S2 and S5, which emerged spontaneously, and revertants S12, S21, S22, S23, which were isolated in irradiation experiments, differed from the wild type strain 49 only in this characteristic. For clone S18, the number of spores was reliably higher than the number of crystals; for S9, crystals reliably prevailed over spores. Among the revertants that did not ferment sucrose, S13 had 1.6 times more spores than crystals, and the effectiveness of spore formation in S17 was 10% lower. Similar differences were found within the group of revertants, in which growth delay on the medium without methionine was additionally revealed.

The conclusion was made from the results of the analysis of R⟶S dissociation that the return to the initial colony morphology occurs, apparently, together with the restoration of the capability to form spores and the normalization of the processes of cell division. The mechanism of emergence of the asporogenous variant R23 is probably not related to the loss of a part of genetic material; some mechanism other than the "switch off–switch on" mechanism underlies the dissociation of strain 49.

DISCUSSION

The results of the research show that S⟶R dissociation of strain 49 *B. thuringiensis* biotype *dendrolimus* results in the formation of variants in colony morphology; their viability is not higher than that of the initial spore-forming strain. These variants would most probably die unless put in conditions which permit their reproduction or restore spore formation. As evident from the clone R5 and as has been noted by other researchers, some of the variants cannot grow even when plated on fresh media. The R variants which survived and were available for analysis stably retained characteristics that were earlier used to identify the cul-

Table 2. Characteristics of the S revertants obtained from R variant R23 of *B. thuringiensis*

Strain and its origin	CFU/ml		Ratio		Hydrolysis of sucrose	Proteolytic activity	Resistance to antibiotics*	Growth delay in the first day on N medium without amino acid **
	before heating	after heating	spores : crystals	χ^2 1 : 1				
R23, spontaneously	0.5×10^8	0	0 : 0	0	–	+/–	–	V; L; A; E; H; I; M; T
S4, spontaneously	5.3×10^8	5.2×10^8	1.5 : 1	27.49	+	+	A, C, M, P	V; L; E; H; I; M; T
S6, spontaneously	4.7×10^8	4.6×10^8	1 : 1.2	4.69	+	+	A, C, M, P	V; L; M
S8, spontaneously	5.9×10^8	5.8×10^8	1 : 1.5	11.77	+	+	A, C, M, P	V; L; M
S9, spontaneously	4.2×10^8	3.9×10^8	1 : 1.3	6.90	+	+	A, C, M, P	V; L
S10, spontaneously	3.9×10^8	3.6×10^8	1.2 : 1	4.39	+	+	A, C, M, P	V; L; M
S13, UVL	5.2×10^8	5.2×10^8	1.6 : 1	20.13	–	+	A, C, M, P	V; L
S14; UVL	4.9×10^8	5.2×10^8	1 : 1	0.76	–	+	A, C, M, P	V; L; M; T
S15, UVL	4.5×10^8	5.6×10^8	1 : 1	0.10	+	+	A, C, M, P	V; L; M
S16, UVL	2.9×10^8	2.6×10^8	1 : 1	3.46	+	+	A, C, M, P	V; L; M
S17, UVL	2.6×10^8	2.3×10^8	1 : 1	3.46	–	+	A, C, M, P	V; L; T
S18, UVL	3.7×10^8	3.5×10^8	1.2 : 1	13.87	+	+	A, C, M, P	V; L
S19, UVL	4.6×10^8	4.6×10^8	1.4 : 1	7.44	+	+	A, C, M, P	V; L; M
S20, UVL	4.8×10^8	7.7×10^8	1 : 1	2.54	+	+	A, C, M, P	V; L; M
S21, UVL	4.3×10^8	4.8×10^8	1 : 1	0.36	+	+	A, C, M, P	V; L
S23,UVL	5.0×10^8	6.2×10^8	1 : 1	0.33	+	+	A, C, M, P	V; L
S24, UVL	5.1×10^8	8.2×10^8	1 : 1	0.01	–	+	A, C, M, P	V; L; M
49, wild type	4.2×10^8	6.7×10^8	1 : 1	1.70	–	+	A, C, M, P	V; L

Notes: * Antibiotics: P, penicillin; A, ampicillin; C, carbenicillin; M, methicillin.

** Valine, V; leucine, L; methionine, M; threonine, T; "+," the presence of activity; "–," the absence of activity or resistance.

tures of the biotype *dendrolimus* isolated from natural sources [15]. These characteristics are probably controlled by obligate genes and they are under the strict control of natural selection. The observed death of a considerable fraction of cells, which is a general characteristic of R variants, may be, in our opinion, the result of this selection. The results of the works mentioned in the introduction [1–9], and of other works, revealed that the features that are mostly changed in dissociants are those important for cell adaptation to unfavorable environmental conditions; most of them could promote their adaptive capabilities. Thus, for example, the appearance of the capacity for sucrose fermentation, registered in the present work in a number of variants, allows them to widen the spectrum of energy sources. Various disruptions of the cell wall, which were observed both microscopically and by their reaction to different combinations of antibiotics of the penicillin group, may provide resistance of substrains to phages to which the initial strain was sensitive [7]. Nevertheless, these features can provide only limited life duration under favorable conditions; restored spore formation is required for the cells to survive the next occurrence of unfavorable conditions.

Investigation of S⟶R and reverse dissociative transfers on the example of an asporogenous variant R23 revealed that reversion to spore formation is possible. Crystal formation is also restored at the same time, although there is no rigid coordination between these processes. It is possible that spore formation after 7–10 days in other R variants reflects this process of inversion; its efficiency depends, apparently, on the mechanism of formation of individual R clones. The published data and our results indicate that the mechanisms of formation of R variants can differ; some variants are not able to restore spore formation, and they die. Thus, additional analysis of 57 R variants isolated from strain 49 for the presence of spores in the course of numerous transfers revealed that six variants did not restore spore formation for two years; their formation was probably

the result of a loss of a part of the initial strain's genetic material.

The experiments carried out by us and by other researchers have shown that the emergence of dissociative forms depends on the medium composition and culture age; it is intensified under unfavorable conditions and occurs only in the stationary phase of growth [3, 4, 16]. In light of these results and the data obtained in the present work, it may be suggested that dissociation of *B. thuringiensis* strain 49 is probably the result of "stationary-phase mutagenesis," which was found in *E. coli* and was recently confirmed for *Bacillus subtilis* [17]. It is believed that asporogenous or oligosporogenic R variants are derivatives of a subpopulation of cells which becoming temporarily hypermutable under stress condition. This population can be formed in the stationary phase of growth in asynchronously developing cultures as a result of the lack of density-dependant signal oligopeptide, triggering the program of sporulation [18], and/or cell transition to the preapoptosis condition due to starvation [19]. When these cells again appear on the nutrient medium, the DNA changes (point mutations, transpositions, realignments) that do not prohibit growth may become fixed in the result of selection (sucrose fermentation, chemotaxis). The changes in the obligate genes, which control the main processes (probably those responsible for somatic O antigen, growth character in the liquid nutrient medium, production of acetylmethylcarbinol, etc.), if not repaired, are most prone to lead to cell death. We are not the only authors to notice the presence of a large number of anomalous and lysed cells in the cultures of R variants [3–9]. The remaining cells will grow until nutrient exhaustion; to survive, they require transfer to optimal nutrient conditions or the restored expression of the genes responsible for the processes of the postexponential phase. Genes *clpC*, *clpP1*, and *clpP2*, found in the strain of the first serotype of *B. thuringiensis* and homologous to the genes of *B. subtilis* controlling the subunits of ATP-dependant proteases may be such genes [20]. At least, the microscopic analysis of the cells of *clp* mutants, for which the authors mention decreased efficiency of sporulation, and our morphological variants of strain 49 revealed a similar picture. As is known, proteins ClpP and ClpC are related not only to stress tolerance, but also to various physiological and morphological developmental processes of many bacteria. For instance, in *B. subtilis*, they are necessary for motility, division, synthesis of the enzymes of degradation, sporulation, and the development of competence.

In conclusion, it can be stated that the process of dissociation of *B. thuringiensis* is the result of genetic events occurring in the stationary phase; they are visualized due to the formation of asporogenous or oligosporogenic variants with colonies of modified morphology. This process occurs within the bounds of the norm of the genotypic reaction and can promote the adaptation of isogenic cultures. The molecular and genetic mechanisms causing dissociation of this bacilli species remain unknown; however, in light of the data obtained, we believe that their understanding is necessary both for successful practical application and for the understanding of the patterns of species existence in biocenoses.

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