DISCOVERY OF A PROTEASE SPECIFICALLY CLEAVING ACTIN IN REVERTANTS OF THE L-FORM OF *Shigella flexneri*

Z. F. Fedorova and S. Yu. Khaitlina

UDC 579.842.15:579.222:577.152.344]
:579.253:[579.252.55:615.33

KEY WORDS: L-forms of *Shigella flexneri*; proteases; antibiotic sensitivity.

Enzymes of L-forms and their revertants of pathogenic bacteria remain at the center of attention of microbiologists and biochemists, for as a result of their study we can come closer to an understanding of the mechanisms of pathogenesis and formation of new varieties of microorganisms, and to visualize the nature of the structural-metabolic transformation which may lie at the basis of L-transformation of bacteria and reversion of L-forms.

The writers showed previously that single cells of *Sh. flexneri* can be converted into L-forms as a result of a single exposure to the 5-nitrofuran derivative, furazolidone [1]. Some revertants of these L-forms were found to be similar in their morphological and cultural properties to bacterial cells of *Escherichia coli* strain A2, which is a producer of a neutral protease specifically cleaving actin, with the formation of a stable fragment with mol.wt. of 36 kD [2-4]. The aim of the investigation was to demonstrate analogous proteolytic activity in cell extracts of eight revertant strains of L-forms of *Sh. flexneri*, exhibiting cultural and morphological similarity with *E. coli* strain A2 and also to compare these strains by their biochemical properties and their sensitivity to antibiotics with different mechanisms of biological action.

EXPERIMENTAL METHOD

Strain *Sh. flexneri* 2a 4115 was isolated from a patient with dysentery [1]. L-forms of the bacteria were induced by a single treatment with furazolidone (0.05 μg/ml in 2% serum-salt nutrient agar) at 28°C [1]. Cultures of revertants of L-forms were obtained on artificial nutrient medium (spontaneous reversion of L-forms in subcultures on aminopeptide agar) or were isolated from noninbred albino mice at different times (5, 16, and 35 days) after experimental reproduction of chronic L-infection (2 and 6 strains, respectively).

Proteolytic activity of the cell extracts was determined by the method used to study activity of cell extracts from strain *E. coli* A2 [4]. The bacteria were cultured on 2% aminopeptide agar (pH 7.2-7.4) at 28 and 37°C without aeration for 1-5 days. The cells were destroyed by ultrasound or by several cycles of freezing and thawing. The extract was clarified by centrifugation at 15,000 rpm for 40 min and added to a solution of actin (or other proteins) with a concentration of 1 mg/ml. The mixture was incubated for 1 h at 20°C and subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. The gels were fixed in a mixture of ethanol and acetic acid and stained with Coomassie G-250. The presence of proteolytic activity was judged by the appearance of a protein zone on the agar, whose mobility was the same as that of the 36-kD fragment of the actin molecule. Electrophoresis in 12% polyacrylamide gel was carried out by the method in [5].

Actin was isolated from rabbit skeletal muscles by the standard method [6]. Tropomyosin from rabbit skeletal muscles was generously provided by N. B. Gusev (Moscow University). Substrate specificity of the protease was determined also by the use of commercial preparations of albumin, casein, and ovalbumin.

First Leningrad Medical Institute. Institute of Cytology, Academy of Sciences of the USSR, Leningrad. (Presented by Academician of the Academy of Medical Sciences of the USSR T. T. Berezov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 110, No. 7, pp. 46-48, July, 1990. Original article submitted January 31, 1989.

0007-4888/90/0007-0901$12.50

TABLE 1. Action of Inhibitors on Activity of Protease from Strains $5a_{2c}$ and *E. coli* A2

Inhibitor	Concentration, mM	Inhibition, %	
		strain $5a_{2c}$	strain E. coli A2
EDTA	1	100	100
L-phenanthroline	5	100	100
Phenylmethylsulfonyl fluoride	1	—	—

TABLE 2. Biochemical Properties of *E. coli* A2 and Revertant Strains

Strain	Feature (fermentation or formation of)															
	glucose	mannose	mannitol	sucrose	lactose	xylose	rhamnose	arabinose	dulcitol	inositol	sorbitol	urea	indole	H_2S	lysine	phenylalanine
A2	+	+	+	+	(+)	±	—	—	—	—	—	—	—	—	—	—
Revertants																
$5a_{2c}$	+	+	+	+	(+)	±	+	+	—	—	—	—	—	—	—	—
IV_{16}	±	±	—	—	—	—	—	—	—	—	±	+	—	—	—	—
9a	+	(+)	—	—	(+)	+	—	—	—	—	±	—	—	—	—	—
VII	—	—	—	—	—	—	—	—	—	—	±	—	—	—	—	—
7	+	+	+	—	(+)	±	+	±	+	—	+	—	+	—	—	—
IV	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—
427	+	+	+	—	—	+	—	—	—	—	(+)	—	—	—	+	—
6	+	+	+	—	+	—	—	—	±	±	±	—	—	—	+	±

Legend. + Fermentation after incubation for 1-2 days, (+) fermentation after 3 days or more, ± variable (different in different subcultures) fermentation: +, (+), or −.

TABLE 3. Sensitivity of *E. coli* A2 and Revertant Strains to Antibiotics and to Furazolidone (MPC, μg/ml)

Strain	Benzylpenicillin	Rystomycin	Ampicillin	Levomycetin	Tetracycline	Erythromycin	Streptomycin	Neomycin	Kanamycin	Gentamicin	Polymyxin	Furazolidone
A2	50	200	50	1,0	2,0	50	5,0	2,5	2,5	0,1	0,1	+
Revertants												
$5a_{2c}$	50	200	50	1,0	2,0	50	5000	2500	5000	0,2	0,1	+
IV_{16}	25	50	8	32	12	25	5000	2500	5000	50	100	—
9a	25	100	0,4	1,0	12	50	5	2,5	2,5	0,2	0,1	—
VII	26	5,0	0,2	8,0	12	50	25	25	25	0,1	50	—
7	50	5,0	2,0	2,0	6,0	50	5	2,5	2,5	0,22	0,3	±
IV	50	200	15	2,0	24,0	25	25	25	25	1,0	0,1	±
427	25	200	4	2,0	5,0	50	25	25	25	1,0	0,3	±
6	50	200	15	4,0	12	50	25	25	25	0,2	0,3	±

Legend. + Marked sensitivity to furazolidone (absence of growth in a concentration of 10-20 μg/ml); ± moderate sensitivity (growth in a concentration of 10-20 μg/ml); − resistance to furazolidone (growth in a concentration of 40 μg/ml).

EXPERIMENTAL RESULTS

To demonstrate proteolytic activity of the preparation of rabbit skeletal muscle, actin was incubated with cell extracts obtained after destruction of bacteria of the initial L-form *Sh. flexneri* and of eight revertant strains. The results of electrophoresis show that the cell extract of the original strain and of seven of the eight cell extracts of revertant strains tested did not induce proteolysis of actin. Only bacteria of strain $5a_{2c}$ (isolated from animals infected with the L-form) contained a protease converting actin into a fragment with mol.wt. of 36 kD.

Degradation of actin by cell extract from bacteria of strain $5a_{2c}$ was inhibited by 5 mM *o*-phenanthroline and by 1 mM EDTA (inhibitors of metalloproteases) but was not inhibited by 1 mM phenylmethylsulfonyl fluoride (inhibitor of serine proteases, Table 1). The results of electrophoresis showed that cell extract $5a_{2c}$, just like protease of *E. coli* A2, does not degrade casein, albumin, ovalbumin, or tropomysin, suggesting that bacteria of strain $5a_{2c}$ synthesize a metalloprotease whose specific substrate is actin.

The biochemical properties of the revertant strains are shown in Table 2. It will be clear from Table 2 that strain $5a_{2c}$ utilizes the same carbohydrates as *E. coli* strain A2, likewise does not decompose urea, does not deaminate lysine and phenylalanine, and does not form indole or H_2S. Unlike the other revertant strains, strain $5a^{2c}$ (like *E. coli* strain A2) hydrolyzes sucrose and does not utilize sorbitol. Unlike *E. coli*, strain A2, strain $5a_{2c}$ can ferment arabinose and rhamnose.

Similarity between strains *Sh. flexneri* $5a_{2c}$ and *E. coli* A2 also was demonstrated when their sensitivity to antibiotics was compared (Table 3). Both strains exhibited marked sensitivity to tetracycline, levomycetin, polymyxin, gentamicin, and furazolidone, and they were resistant to penicillin, ristomycin, ampicillin, and erythromycin. Distinguishing features from the other revertant strains were resistance to ampicillin and sensitivity to furazolidone. Unlike strain *E. coli* A2, strain $5a_{2c}$ also was resistant to streptomycin, kanamycin, and neomycin.

Thus, while exhibiting ability to synthesize the specific protease, the revertant strain of the L-form *Sh. flexneri* $5a_{2c}$ was similar to the producer strain of this protease *E. coli* A2, but different from the other revertant strains in its biochemical properties and sensitivity to antibiotics. Ability to hydrolyze sucrose and inability to utilize sorbitol, resistance to ampicillin, and sensitivity to furazolidone correlate with ability to synthesize protease. These results enable the appearance of proteolytic activity to be interpreted as a marker of profound structural and metabolic changes in the strain under the influence of furazolidone.

Similarity between strains *E. coli* A2 and the revertant strain of the L-form *Sh. flexneri* $5a_{2c}$ indicates that strain *E. coli* A2 may have originated as a result of L-transformation.

The further study of the ability of bacteria and, in particular, of revertants of the L-form of shigellas to synthesize specific protease will be interesting in connection both with the solution to problems of the pathogenicity of shigellas considering that actin is a structural protein of the cell), and also for experimental reproduction of bacteria producing substrate-specific protease.

LITERATURE CITED

1. V. B. Mantulenko, S. Yu. Khaitlina, and N. S. Shelud'ko, Biokhimiya, **48**, No. 1, 69 (1983).
2. Z. F. Fedorova, Antibiotiki, No. 7, 47 (1982).
3. S. Yu. Khaitlina, T. D. Smirnova, Z. F. Fedorova, and A. M. Usmanova, Inventors' Certificate No. 1390241 USSR; Byull. Izobret., No. 15, 12 (1988).
4. S. Yu. Khaitlina (S. Ju Khaitlina), T. D. Smirnova, and A. M. Usmanova, FEBS Lett., **198**, 221 (1986).
5. U. K. Laemmli, Nature, **227**, 680 (1970).
6. D. Pardee and J. A. Spudich, Meth. Cell Biol., **24**, 271 (1982).