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A Chaperonin-Like in the Principal Endocytobiotes of the Weevil *Sitophilus*

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Key words: chaperonin, endocytobiosis, symbiosis, *Sitophilus*, weevil.

Summary: Chaperonins are ubiquitous proteins found in all prokaryotic and eukaryotic cells. They are expressed at high level in several parasitic bacteria and in at least two types of endocytobiosis: in amoebae and in aphids.

In this study, we have investigated the presence of a chaperonin-like, named symbionin, in the principal endocytobiotes of the weevil *Sitophilus*. This protein of 60 kDa (pHi = 6.1) shares a high immunological homology with the groEL chaperonin of *E. coli* and is present in the three symbiotic species of *Sitophilus* (*S. oryzae*, *S. granarius* and *S. zeamais*). In isolated endocytobiotes, the symbionin represents 40% in quantity of the total expressed proteins. These results show the important role of symbionin in the maintenance of the symbiocosme.

Introduction

Chaperonins are ubiquitous proteins which ensure that the folding of polypeptides and their assembly into oligomeric structure occur correctly (Hendrick and Hartl 1993). They are often described in parasitic bacteria because of their high immunogenecity (Shinnick 1991). However, the biochemical function of the chaperonins during the invasion of the host by the bacteria is not known. Considering that endocytobiotes could have evolved from parasitic bacteria (Paillot 1933), it is very interesting to know whether symbiotic organisms still express such a protein or not. It must be pointed out that a chaperonin is expressed at high level in some of the oldest known descendants of endocytobiotes such as mitochondria and chloroplasts (Hemmingsen et al. 1988; Gupta et al. 1989).

In the case of endocytobiosis, a chaperonin-like protein has already been described in two models: the pea aphid *Acyrthosiphon pisum* (Ishikawa 1984) and the amoebae *Amoebae proteus* (Jeon 1983). In these two models, the genes encoding the chaperonin are located downstream of an *E. coli* groES-like gene. Both genes (symL and groEx) are heat shock regulated (Ohtaka et al. 1992; Ahn et al. 1994).

In this study, we have investigated the presence of a chaperonin-like protein in our endocytobiosis model: the weevil *Sitophilus*. This coleoptera (Curculionidae) harbors symbiotic Gram-negative bacteria located in the larval bacteriome and transmitted to the offspring by maternal inheritance (Nardon 1971). The host and the bacteria share very intimate biochemical and genetic interactions. For instance, symbiotes supply the host with vitamins and interfere therefore with its metabolism (Gasnier-Fauchet and Nardon 1987; Wicker 1983). As physiological consequences, we noticed that the symbiotic bacteria increase the fertility and the development rate of the host insect (Nardon and Grenier 1988). Moreover, symbiotic bacteria are completely dependent on the host genome since they cannot divide outside the host cell. They are also involved in an unidirectional cytoplasmic incompatibility phenomenon (Nardon and Grenier 1991 1993).

Four species of *Sitophilus* were studied: *S. oryzae, S. granarius S. zeamais* and *S. linearis*. The weevil chaperonin, called symbionin - in reference to the pea aphid symbionin (Ishikawa 1984) - shows a high immunological homology with the GroEL protein of *E. coli*. Analysis of various biochemical properties (MW, pHi and amino acid composition) leads to classification of this protein within the Hsp60 family. Immunoblotting experiments show that symbionin is overexpressed and accumulated inside the endocytobiotes. Taken together, these observations and those from the literature point out the principal role that symbionin plays in the maintenance of symbiotic equilibrium inside the symbiocosme.

Material and Methods

Insects. *S. oryzae*, *S. granarius* and *S. zeamais* were reared on wheat at 27.5 °C and 75% relative humidity. The fourth instar larvae were taken from inside the grain 21 days after egg laying (28 days for the aposymbiotic strain) and were then homogenized for electrophoresis or fixed for immunohistochemistry.

Insect Medium (MSM). Tris-HCl (20 mM) pH 7.2; Mannitol (250 mM); EDTA (1 mM); $MgCl_2$ (5 mM); KH_2PO_4 (10 mM); KCl (20 mM); GTP (0.5 mM); ATP (2 mM); ADP (2 mM); Pyruvate (5 mM); malate (5 mM); amino acid pool (0,5 mM); BSA (0.4% w/v).

Immunohistochemistry. Fourth instar larvae were fixed in alcoholic Bouin's solution and embedded in paraffin. Sections were mounted on poly-L-lysin coated microscope slides, treated 30 min with 2% H_2O_2 in methanol and probed with anti-symbionin antiserum using Vectastain Elite ABC Kit (Vector). The immunostained sections were then counterstained with 0.1% toluidine blue. For the control, preimmune serum was used instead of anti symbionin antiserum.

Bidimensional Electrophoresis. Isolated endocytobiotes (see isolation method in Heddi 1991) of fourth instar *Sitophilus oryzae* larvae were homogenized (1 ml/bacteriome) in MSM saturated with phenyl-thiourea (PTU). The sample was then desalted by ultrafiltration and resolubilized in solution A: urea (9.5 M); Triton X-100 (16% v/v); dithiothreithol (5% w/v); ampholytes 5/7 (4% v/v); ampholytes 3/10 (2% v/v). The proteins were then isoelectrofocuzed in capillary tubes of 0.5 mm and finally separated by SDS-PAGE.

Immunoblotting. The proteins extracted from endocytobiotes of fourth instar *Sitophilus oryzae* larvae were separated by SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-GroEL antiserum using Vectastain Elite ABC Kit (Vector).

***In vivo* Protein Synthesis and Western Blot Analysis.** Isolated symbiotic bacteria were incubated for 1 h at 27 °C in sterile MSM medium with ^{35}S methionine (Sp. Act.: 1000 Ci/mmol, 50 µCi/ml). They were centrifuged at 8000 g for 10 min and crushed in solution B : Tris-HCl (1.5 M) pH 6.8; dithiothreithol (1,5% w/v); SDS (2% w/v); glycerol (11% v/v); bromophenol blue (75 mg/ml). The neosynthesized labeled proteins were then separated on 10-20% polyacrylamide gel and detected by fluorography. The intensities of protein bands were scanned with the image processing and analysis program *NIH Image* (National Institutes of Health, USA).

Amino Acid Composition. After SDS-PAGE electrophoresis, the symbionin band was transferred to an Immobilon membrane and hydrolysed in 6N HCl containing 1% thioglycolic acid at 120 °C for 24 h. Amino acids in the hydrolysate were resolved through an autoanalyzer Beckman 6 300.

Results

Immunohistochemistry of Cross Sections

The use of a polyclonal antibody against the chaperonin GroEL of *E. coli* (Hara et al. 1990) allowed us to reveal the presence of symbionin in all symbiotic strains of *Sitophilus* (*S. oryzae, S. granarius* and *S. zeamais*). The specific coloration of the antibody was found in the larval bacteriome and in the apical bacteriome of the adult female ovaries (Fig. 1A, B). The other tissues were free of symbionin. Figure 1C shows an enlargement of a *S. granarius* ovocyte with some specifically stained endocytobiotes.

This prokaryotic protein was not found in the aposymbiotic species *S. linearis* nor in the heat-treated aposymbiotic strain of *S. oryzae*.

Biochemical Characterization

Whole proteins of isolated endocytobiotes were extracted and separated on a bidimensional electrophoresis gel (Fig. 2). A molecular weight of 57 kDa and a pHi of about 6.1 were found for the *Sitophilus oryzae* symbionin. Immunoblotting experiments (Fig. 3) also confirm the presence of symbionin inside the endocytobiotes. Finally, the amino acid composition of the protein was determined and compared with those of three other chaperonins: GroEL of *E. coli*, symbionin of

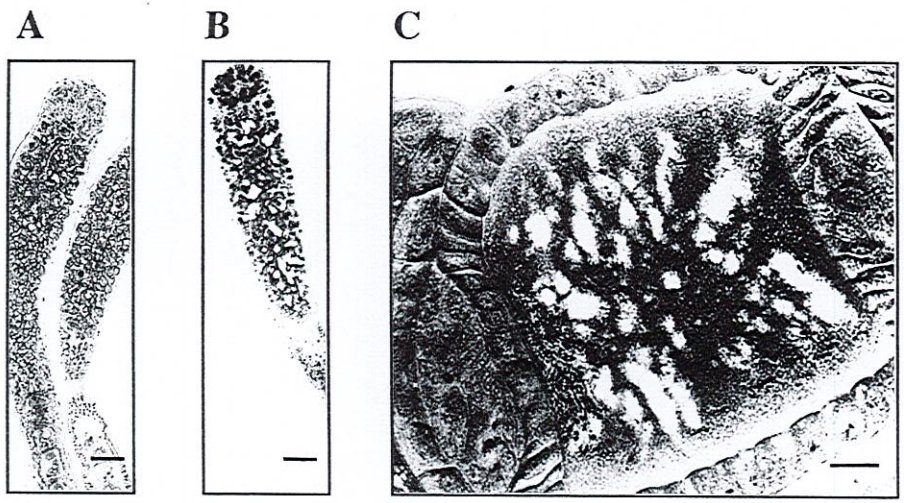

Fig. 1. Immunohistochemistry of *Sitophilus granarius* cross sections. **A** and **B** Adult ovaries and their apical bacteriome; bars = 100 µm. For the control **A**, preimmune serum was used instead of anti-symbionin antiserum. **B** The presence of symbionin was visualized in the dark inside the bacteriome **B**. **C** An enlarged image of an ovocyte with immunostained endocytobiotes at the posterior pole; bar = 10 µm)

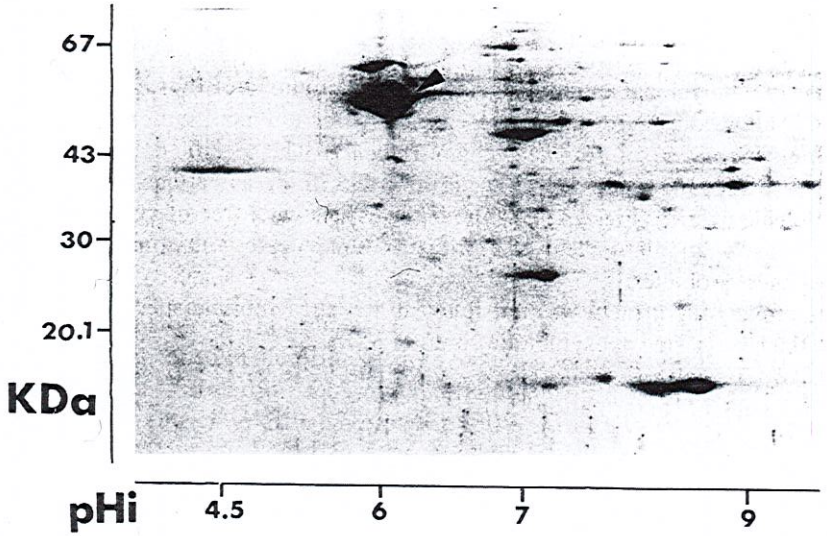

Fig. 2. Bidimensional electrophoresis. Proteins of isolated endocytobiotes of fourth instar *Sitophilus oryzae* larvae were separated on bidimensional electrophoresis gel. The symbionin is visualized on the gel (see arrow head)

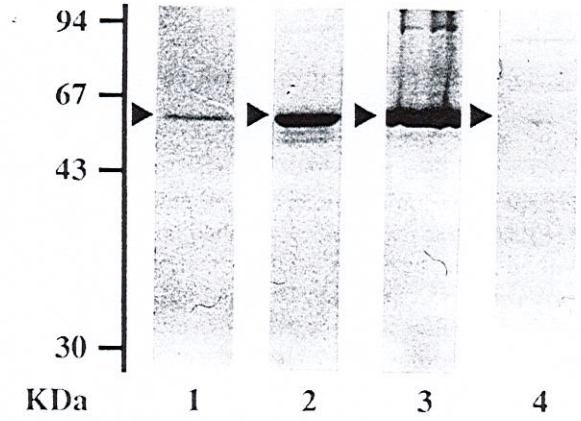

Fig. 3. Immunoblotting. The extracted proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-GroEL antiserum. *1* Whole proteins of the symbiotic strain of *S. oryzae*; *2* proteins of isolated bacteriocytes; *3* proteins of isolated endocytobiotes; *4* whole proteins of the aposymbiotic strain of *S. oryzae*.

Acyrthosiphon pisum endocytobiotes and GroEx of *Amoebae proteus* endocytobiotes (Table 1).

In vitro Neosynthesized Proteins

The isolated endocytobiotes cannot divide outside their host, but they can survive for more than 1 month in MSM medium. Their viability was assessed under the microscope by the movement of the bacteria (Fig. 4). From this analysis it was found that a large part of the bacterial metabolism *in vitro* is focused on the production of symbionin. The band corresponding to symbionin represents about 40% of total neosynthesized proteins but the purpose of this important energetic cost for the bacteria is still not understood.

Discussion

All the studied symbiotic species of *Sitophilus* harbor endocytobiotes expressing symbionin at a high level. This protein shares the biochemical characteristics of the Hsp60 protein family. Its molecular weight is 57 kDa and its pHi is about 6.1. Moreover, its global amino acid composition is very close to that of other chaperonins.

As symbionin is expressed at a high level inside the symbiotic bacteria, we can thus assume that it has an important function in the symbiotic relationships. The most interesting point is to understand why this protein is produced by the bacte

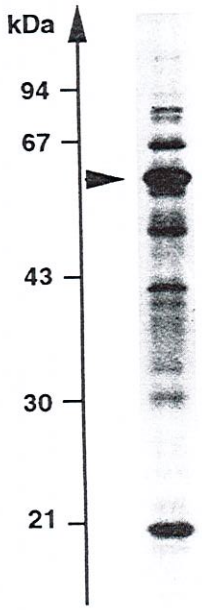

Fig. 4. Endocytobiotes of *S. oryzae*. Electrophoresis of *in vitro* neosynthesized proteins. Symbiotic bacteria were, directly after isolation, incubated for 1 h at 27 °C in sterile MSM medium with ^{35}S methionine. After electrophoresis, the intensities of protein bands were scanned with the *NIH-image* program

Table 1. Amino acid compositions (% mol) of *S. oryzae* symbionin. Comparison between the amino acid composition of *S. oryzae* symbionin and three other chaperonins: GroEL (*E. coli*), symbionin (*Acyrtosiphon pisum* endocytobiotes) and GroEx (*Amoeba proteus* endocytobiotes). The amino acid composition of the three chaperonins were found in Swiss prot database

	S. oryza symbionin	*E. coli* GroEL	*A. pisum* symbionin	*A. proteus* GroEx
Ala	8,7	13,7	10,8	12,36
Arg	3,9	4	6,8	4,91
Asx	11,2	11,8	11,2	9,82
Glx	10,9	9,8	8,8	11,27
Gly	12	10,4	10,5	10,55
His	1,7	0,2	0,7	0,73
Ile	5,5	6	6,7	6,73
Leu	8,7	7,5	9,3	6,55
Lys	7,2	7,3	8,3	7,09
Met	2	4	4,3	5,27
Phe	3,2	1,3	1,5	1,82
Pro	5,15	2,6	1,5	2,00
Ser	6,2	3,1	5,1	4,55
Thr	4,5	6	3,5	5,82
Tyr	2,4	1,3	1,9	0,91
Val	6,9	10,6	9,3	9,09

ria. The presence of such a protein in parasitic bacteria as well as in three models of endocytobiosis (the weevil, the pea aphid and amoebae) corroborate with a basic function, conserved during evolution, in the maintenance of the symbiotic equilibrium.

Finally, we have formulated four hypotheses on the possible function of the symbionin in the weevil: (1) the symbionin could be a storage protein, the host could then use it by lysing its own endocytobiotes; (2) the symbionin is expressed to fold and assemble the endocytobiote's own proteins; (3) the symbionin could fold and assemble some host proteins imported from the host cytosol, these proteins could be used by the bacterial machinery or re-exported in the host cytosol afterwards; (4) the protein syntheses could be induced by the host, and the significant energetic cost for the bacteria could inhibit its growth potential. By this means, the host could control the bacterial division and preserve itself from invasion by the bacteria.

Currently, the two first hypotheses do not seem very probable (Charles et al. 1995), but it is still not an obvious choice between the two last hypotheses. In one case, the protein seems to be beneficial to the bacteria, and in the other case to the host insect. We are investigating the biochemical mechanism of the symbionin in one way and the expression and the regulation of its encoding gene in another way. These experiments will surely help us to better understand this fascinating model.

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