# Presence of a protein specific of endocytobiosis (symbionin) in the weevil Sitophilus

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# Presence of a protein specific of endocytobiosis (symbionin) in the weevil *Sitophilus*

## *Présence d'une protéine spécifique de l'endocytobiose (symbionine) chez le charançon* Sitophilus

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### RÉSUMÉ

Les chaperons sont des protéines ubiquistes présentes dans tous les types cellulaires procaryote et eucaryote. Elles sont surexprimées chez de nombreuses bactéries parasites et sont impliquées dans au moins 2 cas d'endocytobiose décrits jusqu'à présent : chez l'amibe et chez le puceron. La présente étude montre qu'une protéine appelée symbionine, possédant une homologie immunologique avec la protéine GroEL d'*E. coli*, intervient dans la relation symbiotique de 3 espèces de *Sitophilus (S. oryzae, S. granarius* et *S. zeamais)*. Cette protéine n'est, en revanche, pas retrouvée dans l'espèce naturellement asymbiotique *S. linearis* ni dans la souche aposymbiotique de *S. oryzae* obtenue au laboratoire. La symbionine est stockée en quantité importante à l'intérieur des endocytobiotes et l'analyse de sa composition en acides aminés semble confirmer son appartenance à la famille des chaperons plutôt qu'à une des familles de protéines de stockage décrites auparavant dans la littérature chez les insectes. ▲

***Mots clés :*** *chaperon, endocytobiose, symbiose,* Sitophilus, *charançon.*

### ABSTRACT

*Chaperonins are ubiquitous proteins found in all prokaryotic and eukaryotic cells. They are overproduced in several parasitic bacteria and are implicated in at least 2 types of endocytobiosis : in amoebae and in aphids. This work puts in evidence that a protein named symbionin, which shows an immunological homology with the* E. coli *protein GroEL, is present in the symbiotic relationship of 3 species of* Sitophilus (S. oryzae, S. granarius, *and* S. zeamais). *This protein is neither found in the naturally asymbiotic specie* S. linearis *nor in the aposymbiotic strain of* S. oryzae *obtained in the laboratory. This symbionin is stored in a great quantity within endocytobiotes and its amino acid composition seems to corroborate its chaperonin resemblance rather than its possible function as one of the insect storage proteins already described in the literature.* ▲

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

### VERSION ABRÉGÉE

Paillot [1], en 1933, a été parmi les premiers à considérer la symbiose comme le résultat de la coadaptation et de la coévolution de 2 partenaires initialement engagés dans une relation de type hôte/parasite. S'il est maintenant certain que des facteurs génétiques d'interactions sont nécessaires à l'établissement et au maintien de l'équilibre symbiotique, leur nature et les mécanismes biochimiques mis en jeu demeurent mal connus. La production de protéines de choc thermique en réponse au stress imposé par les conditions de vie intracellulaire est un phénomène très couramment observé chez les bactéries parasites. Dans le cas de l'endocytobiose, c'est-à-dire de la symbiose intracellulaire, l'implication d'une molécule chaperon produite en grande quantité par le génome bactérien a été décrite dans 2 modèles : le puceron du pois (Acyrthosiphon pisum) et l'amibe (Amoeba proteus).

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Chez *A. pisum*, il a été montré que les endocytobiotes, *in vivo*, synthétisent préférentiellement une seule protéine appelée symbionine. Cette protéine, qui semble avoir un rôle important dans la relation symbiotique, appartient à la famille des chaperons moléculaires et possède une grande homologie avec la protéine GroEL d'*E. coli*. Elle manifeste *in vitro* une activité de molécule chaperon mais *in vivo* son rôle dans les mécanismes d'interactions hôte/symbiote n'est pas encore déterminé précisément. Chez l'amibe, l'endocytobiose est très récente et parmi les protéines synthétisées en quantité importante par les endocytobiotes figure également un chaperon.

Dans cette étude, la présence d'une symbionine a été recherchée chez le genre *Sitophilus* qui présente une endocytobiose très particulière puisque, bien qu'elle soit très intégrée, elle reste non obligatoire. Quatre espèces de *Sitophilus* ont été étudiées : 3 espèces symbiotiques, *S. oryzae, S. granarius* et *S. zeamais* et 1 naturellement asymbiotique, *S. linearis*. Une souche de *S. oryzae* aposymbiotique obtenue au laboratoire par traitement à la chaleur a également été utilisée. Rappelons la grande importance de la symbiose chez *Sitophilus*, où, en accroissant la vitesse de développement et la fertilité, elle conditionne en grande partie la nuisibilité de ces insectes pour les céréales entreposées.

Un marquage immunologique sur coupes microscopiques (réalisé grâce à un anticorps polyclonal anti-GroEL) a permis de montrer qu'une symbionine est présente en grande quantité dans les bactériomes larvaires et ovariens, et plus précisément dans les endocytobiotes de 3 espèces symbiotiques de *Sitophilus*. En revanche, cette protéine n'a pas été retrouvée dans les 2 espèces aposymbiotiques.

Afin de confirmer la localisation de cette protéine, des marquages sur profil d'électrophorèses ont été réalisés. Il a ainsi été possible de démontrer que la symbionine est bien essentiellement stockée dans les endocytobiotes. L'expression de chaperons semble donc une propriété commune aux divers types de symbiotes intracellulaires, des plus récemment acquis comme les bactéries parasites aux plus anciens comme les chloroplastes et les mitochondries. Et si ce principe a été si bien conservé par l'évolution, il faut logiquement s'attendre qu'il ait un rôle important dans le maintien de l'équilibre hôte/symbiote.

Mais, chez le charançon, il est légitime de se demander pourquoi les endocytobiotes synthétisent en quantité si importante une molécule chaperon alors qu'ils ne se divisent que très peu dans le bactériome. Un élément de réponse serait que la symbionine soit une protéine de stockage que l'hôte assimilerait en digérant directement ses endocytobiotes. Les figures de lyse observées au niveau des bactériomes de l'apex des ovarioles chez l'adulte pourraient venir conforter cette hypothèse.

En vue de déterminer la fonction de cette protéine, sa composition en acides aminés a été déterminée et comparée à celles d'autres protéines grâce à une analyse factorielle des correspondances. Cette analyse a été réalisée à partir du tableau des compositions de 19 protéines : 9 chaperons moléculaires, 6 protéines de stockage de *Sitophilus* et 4 protéines de stockage trouvées chez d'autres insectes. Ces résultats appuient l'hypothèse de l'appartenance de la symbionine de *Sitophilus* à la famille des chaperons, caractérisée par des acides aminés hydrophobes tels que la valine, la leucine et l'isoleucine. Il faut néanmoins noter que la symbionine de *S. oryzae* semble se démarquer des autres molécules chaperons par des taux plus élevés de proline et d'histidine et des taux plus faibles de valine et d'isoleucine. Et s'il est effectivement peu probable que la symbionine de *Sitophilus* possède un simple rôle de protéine de stockage, il serait tout à fait concevable qu'elle joue un rôle dans les échanges bactéries/insectes. C'est donc bien vers sa fonction de molécule chaperon qu'il conviendra d'orienter les recherches futures afin de confirmer ou d'infirmer son originalité apparente.

Le modèle *Sitophilus* présente un type d'endocytobiose très intégré mais qui possède toutefois l'avantage de n'être pas strictement obligatoire. L'ensemble de ces résultats montre qu'une symbionine est impliquée dans la relation symbiotique comme c'est le cas dans les endocytobioses obligatoires. Ce modèle apparaît donc particulièrement intéressant dans le cadre de l'étude des interactions hôte/symbiote à un niveau moléculaire. ▲

According to the theory of Paillot [1], symbiotes are probably parasitic bacteria which have been domesticated afterwards. From an evolutionary point of view, endocytobiosis (or intracellular symbiosis) must be considered as the result of the coadaptation and the coevolution of the associated partners leading to the formation of the symbiocosme [2]. The amoeba endocytobiosis, which accidentally appeared in Jeon's laboratory [3], is a very good illustration of this process. The result of this domestication is generally the lost of independence for the 2 partners, endocytobiotes being gradually transformed in cellular organelles [2, 4]. It is obvious now that some genetic interactive factors are needed to initiate and maintain symbiotic association [2, 5], but their identity and their biochemical mechanisms still remain unclear. Heat shock protein production is well described in parasitic bacteria [6]. In the case of endocytobiosis, a chaperonin is implicated and has been described in 2 models : the pea aphid, *Acyrthosiphon pisum* [7], and the amoeba, *Amoeba proteus* [8].

In *A. pisum*, endocytobiotes preferentially synthesize *in vivo* one protein of 63KDa named symbionin [7]. This protein belongs to the chaperonin's family and possesses a 86% amino acid homology with the *E. coli* protein GroEL [9]. The selective expression of this protein and its high degree of conservation led the authors to think that symbionin could have an important role in symbiotic relationships. In *A. proteus*, the endocytobiosis is very recent and among the proteins expressed *in vivo* by endocytobiotes, a chaperonin has been found [10].

In *Sitophilus*, endocytobiotes are located in a larval bacteriome, in anterior mesenteric caeca of young adults and in female germ cell [11]. Bacteria are very highly integrated in the host metabolism. They induce the differentiation of specialised cells named bacteriocytes, interfere with the host methionine metabolism and supply the weevil with several vitamins [12, 13]. As a consequence, they increase the fertility and the speed of development and are greatly responsible of the harmfulness of *Sitophilus* against stored cereals [14, 15]. Despite these facts, symbiosis is not obligatory and viable aposymbiotic weevils have been obtained by heat treatment [16].

In this study, we have looked for the existence of a symbionin in 4 species of *Sitophilus (S. oryzae, S. granarius, S. zeamais* and *S. linearis)* by using immunohistochemistry, immunoblotting and amino acid detection.

## Materials and methods

### Insects

*S. oryzae, S. granarius* and *S. zeamais* were reared on wheat at 27.5°C and 75% relative humidity [17]. *S. linearis* was reared on dried tamarind pods. The aposymbiotic strain of *S. oryzae*, obtained by heat shock, was that described by Nardon [16]. The fourth instar larvae were taken from inside the grain 21 days after egg laying (28 days for the aposymbiotic strain) and were then crushed for electrophoresis or fixed for immunohistochemistry.

#### Antiserum

Antisera against the GroEL protein of *E. coli* was prepared in male Japan White Rabbits as previously described [18].

#### Immunohistochemistry of paraffin tissue sections

Fourth instar larvae were fixed in alcoholic Bouin's solution and embedded in paraffin. Five micrometer thick sections were mounted on poly-L-lysin coated microscope slides. The sections were treated 30 min with 2% $H_2O_2$ in methanol (elimination of endogenous peroxidase) and then probed with anti-symbionin antiserum using Vectastain Elite ABC Kit (Vector) as described in [19]. The immunostained sections were then counterstained with 0.1% toluidin blue. For the control, preimmune serum was used instead of anti-symbionin antiserum.

#### Electrophoresis

The following insect media have been used:
Y medium: 10 g NaCl, 1.5 g KCl, 0.5 g $CaCl_2$, 0.18 g $NaHCO_3$, 0.01 g $NaH_2PO_4$, 1 g Glucose, 0.4 g levulose, 15 g Maltose, qsp 1 l $H_2O$, pH = 6.8 and PO = 300 mOsm. Solution A: 1 µg/ml PMSF, 0.6 µg/ml EDTA, 0.2 µg/ml Pepstatin, 0.2 µg/ml antipain, saturation Phenylthio-uree in buffer Y. Solution B: Tris-HCl buffer (pH = 6.8), 15 mg/ml Dithiothreitol, 2% (w/v) Sodium Dodecyl Sulfate (SDS), 10% (v/v) Glycerol, 75 µg/ml bromophenol blue.
Insect tissue was homogenized at 4°C in solution A at 25 µl/mg. After centrifugation (800 g, 3 min), the supernatent proteins were precipitated in 5 volumes of acetone at – 30°C for 12 h. The proteins were then centrifuged (9,000 g, 10 min), dried and solubilized in solution B. The samples were finally boiled at 100°C for 3 min, centrifuged (5,000 g, 5 min) and a volume equivalent to 1 mg of wet insect (50 µg of protein) was subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) using 12% homogeneous separating gel [20].

#### Immunoblotting

After electrophoresis, the proteins were transferred to nitrocellulose membrane (Bio Bind-NC, 0.2 µm, Whatman) in transfer buffer (0.05 M Tris, 0.05 M orthoboric acid) with a transfer blot apparatus (Bio Rad) at 40V for 4 h 30. Membranes were then saturated with skim powdered milk (Régilait®) at 37°C for 12 h. The band due to symbionin was probed with anti-symbionin antiserum using Vectastain Elite ABC Kit (Vector) as described in [19], except that the NaCl concentration of the ABC reagent was increased at 0.45 M and the peroxidase substrate was 0.4% Diaminobenzidine, 0.1% $H_2O_2$ in distilled water. For the control preimmune serum was used instead of antiserum.

#### Amino acid analysis

Endocytobiotes and mitochondria of *S. oryzae* were isolated as described in [21], the proteins were separated by SDS-PAGE and transferred to Immobilon membrane (Immobilon-P, 0.45 µm) at 40V for 3 h with transferred Blot apparatus (Bio Rad). Proteins were then stained (1 mg/ml aminoblack, 7% (v/v) acetic acid, 45% (v/v) methanol) and the band corresponding to symbionin was cut and hydrolysed in 6N HCl containing 1% thioglycolic acid at 120°C for 24 h. Amino acids in the hydrolysate were resolved through a autoanalyzer Beckman 6300.

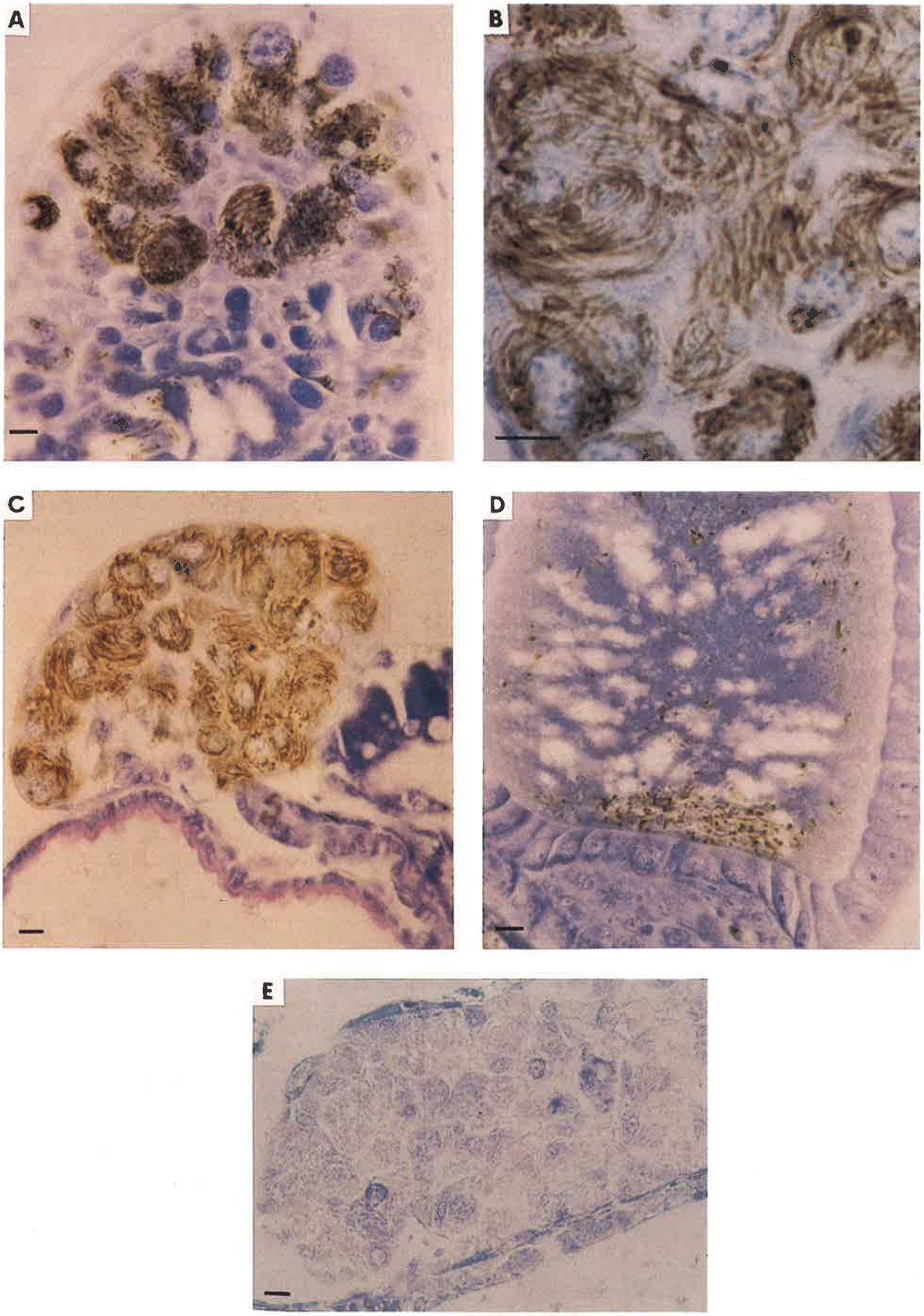
## Results

#### Immunohistochemistry of cross sections

*Figure 1* represents cross sections of *Sitophilus* tissues that were immunostained with anti-GroEL antiserum. Each slide was compared to its control probed with preimmune serum in order to avoid non specific binding interpretations. *Figure 1A* and *B*, show a *S. granarius* ovary with its apical bacteriome densely stained. In the larvae of the same species *(Fig. 1C)*, the bacteriome is also very intensively stained compared to the control *(Fig. 1E)*. No other tissue showed any sign of the presence of symbionin. *Figure 1D* shows an enlarged image of a *S. granarius* ovocyte with some specifically stained endocytobiotes at the posterior pole. Symbionin is therefore present inside the endocytobiotes even if they are outside the bacteriome. The same observation can be made with the 2 other symbiotic species of *Sitophilus*: *S. oryzae* and *S. zeamais* (data not shown). Judging from the density of immunostaining, the *S. granarius* contains more symbionin (or a form with higher affinity for the antiserum) than the other species. No symbionin is found in the aposymbiotic strain of *S. oryzae* (data not shown). Cross sections of *S. linearis* (a naturally asymbiotic species) were stained and no specific binding was observed neither in the larvae nor in the adult (data not shown).

#### Immunoblotting

In order to specify the location of the *S. oryzae* symbionin, the proteins of different insect compartments were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-symbionin antiserum. The results are presented on *Figure 2*. For each lane, control with preimmune serum was made. Symbionin is found in the whole proteins of the symbiotic strain (lane 1), in the bacteriomes (lane 2) and especially in isolated endocytobiotes where it is in a very large quantity (lane 3) meaning that symbionin is probably stocked inside the endocytobiotes.
A very thin band is observed in the whole proteins of the aposymbiotic strain (lane 4). This band is certainly due to the mitochondrial heat shock protein (hsp60m). The high affinity of the antiserum for hsp60m has been verified in other experiments (data not shown).


A
B
C
D
E

◀ Figure 1. ***Immunohistochemistry of* Sitophilus granarius *adult and larvae cross sections.*** *(A) An adult ovary and its apical bacteriome. The presence of symbionin was visualised in brown inside the bacteriome.* ***(B)*** *An enlarged image of an apical bacteriome with its bacteria specifically stained.* ***(C)*** *A larval bacteriome.* ***(D)*** *An enlarged image of an ovocyte with immunostained endocytobiotes at the posterior pole.* ***(E)*** *The control (larval bacteriome) was probed with preimmune serum instead of anti-GroEL antiserum. All the sections were counterstained with Toluidin Blue. Scale bars: 10 µm.*

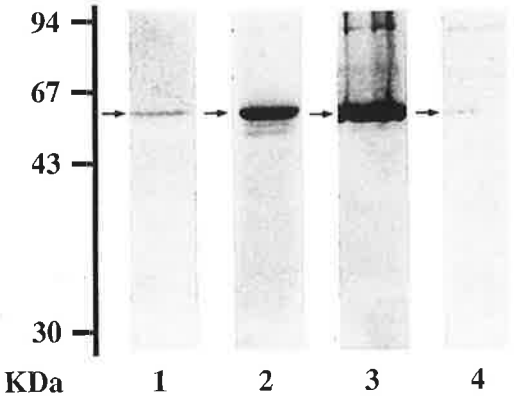



Figure 2. ***Immunoblotting and localisation of* S. oryzae *symbionin.*** *The extracted protein were separated by SDS-PAGE, transferred on nitrocellulose membrane and probed with anti-GroEL antiserum. (1) Whole proteins of the symbiotic strain of* S. oryzae*; (2) proteins of isolated bacteriocytes; (3) proteins of isolated endocytobiotes; (4) whole proteins of the aposymbiotic strain of* S. oryzae.

Table I
Amino acid composition (%mol) of *S. oryzae* symbionin and hsp60m

| | Symbionin *S. oryzae* | hsp60m *S. oryzae* |
|---|---|---|
| Asx | 10,9 | 10,2 |
| Thr | 4,5 | 4,4 |
| Ser | 6,2 | 5,3 |
| Glx | 11,2 | 12 |
| Pro | 5,15 | 2,2 |
| Gly | 12 | 13,6 |
| Ala | 8,7 | 11,6 |
| Val | 6,9 | 8,1 |
| Met | 2 | 3,5 |
| Ile | 5,5 | 5,1 |
| Leu | 8,7 | 7,8 |
| Tyr | 2,4 | 1,8 |
| Phe | 3,2 | 2,1 |
| Lys | 7,2 | 7,2 |
| His | 1,7 | 1,2 |
| Arg | 3,9 | 3,9 |

### Amino acid composition

The proteins of isolated endocytobiotes (and mitochondria) of *S. oryzae* were separated by SDS-PAGE; the bands corresponding to symbionin (and hsp60m) were cut and subjected to analyze in an amino acid analyzer. Results given in %mol are presented in *Table I*. This method of extraction does not ensure a very high degree of purity of the analyzed proteins, but as it can be seen below, the determination was sufficient for the multivariate analysis which was realised.

### Multivariate analysis

Considering the presence of high quantity of stored symbionin in the weevil, it was interesting to compare the amino acid composition of this protein with some of other insect storage proteins described in the literature. The compositions of 7 chaperonins found in endocytobiotes or parasitic bacteria, 6 storage proteins found in *Sitophilus* and 4 other species storage proteins were chosen to make the comparison by factorial correspondence analysis. The 2 superposed factorial maps (axes 1-2), representing 72% of total inertia, are presented on *Figure 3*. Three groups of proteins can be distinguished by this analysis. The first group, correlated with hydrophobic amino acids like alanine, valine, leucine and isoleucine, is the group of chaperonins. It includes both *S. oryzae* symbionin and hsp60m. The second group, with tyrosine and serine, includes the *Sitophilus* storage proteins named tyrostaurines [22]. The third group is correlated with phenylalanine and histidine and corresponds to *Bombyx mori* and *Calliphora* storage proteins. Two proteins, B30K and Sor-ary are both rich in phenylalanine and tyrosine and form an intermediate group of storage proteins between 2 and 3. It is also noteworthy that the *S. oryzae* symbionin is far from the inertia centre of the chaperonin group compared to the other members. It also possesses more proline and histidine residues and less valine and isoleucine.

## Discussion

Chaperonins are ubiquitous proteins found in all cellular types and especially in parasitic bacteria [6]. Considering that endocytobiotes could have evolved from parasitic bacteria [3], it is thus not very surprising to find that they contain a chaperonin. But the most interesting question is to know whether the presence of symbionin is a general process of endocytobiosis conserved during evolution. It must be pointed out that a chaperonin is expressed in great quantity in some of the oldest endocytobiotes known as mitochondria and chloroplasts [23, 24].

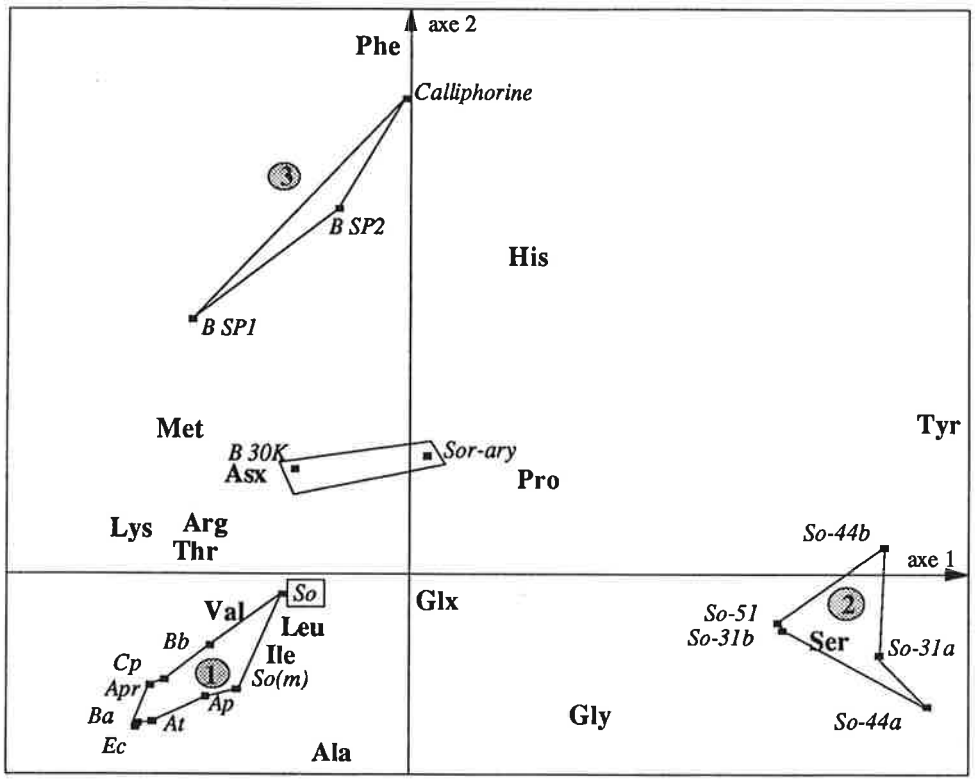



Figure 3. ***Factorial correspondence analysis (FCA) of different insect storage proteins and chaperonins.*** *The analysis was based on the amino acid composition (%mol) of 19 proteins. The 2 factorial maps were superposed so as to visualise the amino acids characteristic of each of the 3 groups of proteins. So:* S. oryzae *symbionin(*); So(m):* S. oryzae *hsp60m(*); Ap:* A. pisum *symbionin [9], Apr:* Amoeba proteus *symbionin [10], At :* Agrobacterium tumefaciens *chaperonin($); Ec:* Escherichia coli *GroEL($); Ba:* Brucella abortus *chaperonin($); Cp:* Chlamydia pneumoniae *chaperonin($); Bb:* Borrelia burgdorferi *chaperonin($); B SP1, B SP2, B 30K:* Bombyx mori *storage proteins [25], Calliphorine:* Calliphora *storage protein [26], Sor-ary, So-31a, So-31b, So-44a, So-44b, So-51:* Sitophilus *storage proteins [22].*
*(*) Determined in the laboratory.*
*($) Swiss prot data base.*

This study helps us to answer the previous question; in fact it shows that all the tested symbiotic species of *Sitophilus* harbour endocytobiotes producing symbionin *(Fig. 1).* On the opposite, none of the 2 aposymbiotic species produces any chaperonin in so large quantity. In the case of parasitic bacteria, the authors generally propose that heat shock proteins are produced in order to protect bacteria from the host aggression, but in the case of endocytobiosis it is even less clear.

In *A. pisum*, symbionin is produced inside the bacteria and does not seem to be exported outside, meaning that it is used for the endocytobiotes own sake [19]. The results obtained with *S. oryzae (Fig. 2)* are in agreement with this hypothesis as symbionin seems to be stocked inside bacteria.

But in the case of *Sitophilus*, an interesting problem is to know how such a quantity of chaperonin is produced by endocytobiotes when they divide very slowly in the bacteriome. The simplest hypothesis would be to consider this peculiar chaperonin as a storage protein, the weevil being able to ingest it by lysing its own endocytobiotes. Pictures of lysed bacteria that have ever been described in the adult ovary, could argue in this sense [11]. In this way, the amino acid composition of *Sitophilus* symbionin was compared with that of storage proteins described in the literature [22]. The analysis *(Fig. 3)* puts in evidence that *Sitophilus* symbionin does not present a characteristic storage protein composition. Its place on the factorial map near hydrophobic amino acids, representative of the chaperonin family, seems to confirm its chaperonin identity. But it is noteworthy that symbionin is the only chaperonin being so far from the inertia group's centre. Considering that the composition of *Sitophilus* symbionin and hsp60m were determined simultaneously and that hsp60m does not show any of these particularities, the hypothesis of polluting proteins extracted with the electrophoresis band can be rejected. If these preliminary results suggest that *Sitophilus* symbionin could have evolved differently from other chaperonins, only molecular biology studies will be able to solve this problem.

The *Sitophilus* symbiosis was qualified as integrated endocytobiosis by Nardon in 1993 [2], and despite this fact it presents the peculiar advantage of being non obligatory. This study has shown that, as it is the case in obligatory endocytobiosis, a chaperonin is implicated in the host/symbiote relationships. It is conceivable that this protein could be implicated in the very intimate exchanges taking place between the 2 associated partners. Thus studies of the *in vitro* biochemical properties of the *Sitophilus* symbionin appear to be of particular interest. ▼

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