Application under article 17 of Regulation 1829/2003

PT73 (TM)

**Dried killed bacterial biomass,
by-product of L-Threonine production by fermentation
using a genetically modified strain of *E. coli* K12,
intended to be used as feed material**

PART II

Summary

**Dossier prepared using, as guidance,
the 'Guidance Document for the risk assessment
of genetically modified micoorganisms and
their derived products intended for food and feed use'
(adopted on 17 May 2006, The EFSA Journal 374)**

AJINOMOTO EUROLYSINE S.A.
April 2008

SUMMARY OF APPLICATION FOR THE GM PRODUCT PT73 *E.COLI* (THR) FOR FEED USE

A. GENERAL INFORMATION

1. Details of application

a) Member State of application France
b) Application number Not yet allocated to the Applicant at the time of the remittance of the dossier to the French competent authorities.
c) Name of the product (commercial and other names) -For the purpose of this dossier: PT73 (TM) -Commercial name: PROT-AEL-T (However, subject to the confirmation that it may be acceptable as registered trade mark)
d) Date of acknowledgement of valid application Validity of the application to be established by EFSA.

2. Applicant

a) Name of applicant Ajinomoto Eurolysine S.A.S, contact person:
b) Address of applicant 153, rue de Courcelles 75817 PARIS Cedex 17 France
c) Name and address of the person established in the Community who is responsible for the placing on the market, whether it be the manufacturer, the importer or the distributor, if different from the applicant (Commission Decision 2004/204/EC Art 3(a)(ii)) The person established in the Community responsible for the placing on the market will be the Applicant.

3. Scope of the application

☐ GM microorganisms and/or derived products for food use

■ GM microorganisms and/or derived products for feed use

☐ GM microorganisms and/or derived product(s) belonging to Group 1, as defined in Chapter II, 2. of this Guidance

■ GM microorganisms and/or derived product(s) belonging to Group 2, as defined in Chapter II, 2. of this guidance

☐ GM microorganisms and/or derived product(s) belonging to Group 3, as defined in Chapter II, 2. of this guidance

☐ Import and processing (Part C of Directive 2001/18/EC)

4. Is the product being simultaneously notified within the framework of another regulation?

Yes ☐	No ■
If *yes*, specify	

5. Has the GM microorganism been notified under Part B of Directive 2001/18/EC and/or Directive 90/220/EEC?

Yes ☐	No ■
If *no*, refer to risk analysis data on the basis of the elements of Part B of Directive 2001/18/EC	

6. Has the GM microorganism or derived products been previously notified for marketing in the Community under Part C of Directive 2001/18/EC or Regulation (EC) 258/97?

Yes ☐	No ■
If *yes*, specify	

7. Has the product been notified in a third country either previously or simultaneously?

Yes ☐	No ■
If *yes*, specify	

8. General description of the product

a) Name of the recipient or parental microorganism and the intended function of the genetic modification

The product PT73 (TM), subject of the present application, consists of the dried killed cells of a genetically modified strain of *Escherichia coli* K-12 (*E. coli* K-12), named strain AG3139. The strain AG3139 is used by the Applicant for the production of L-threonine by fermentation of substrates of agricultural origin.

Strain AG3139 has been constructed from a specific strain of *E. coli* K12 – strain MG1655 - in several steps using conventional and modern techniques of genetic modifications. The purpose of the genetic modifications is to obtain a high production rate of L-threonine.

b) Types of products planned to be placed on the market according to the authorization applied for

The product PT73 (TM) (dried killed bacterial biomass) mentioned in a), will be a by-product of the L-threonine manufacturing process using strain AG3139.

c) Intended use of the product and types of users

Increase in the production of L-threonine has resulted in an increased tonnage of bacterial biomass recovered after completion of the fermentation phase. Therefore, a new outlet had to be found for this by-product.

Considering its high nitrogen content, this dried killed bacterial biomass may serve as a direct or indirect source of protein for animals. Therefore, a use as feed material - a concentrated source of crude protein - for compound feedingstuffs formulated for pigs, salmonids and ruminants (inter alia dairy cows) is found.

The product will be sold in pellet form (minimization of dust production) and in 'bulk' to feed mills only.

d) Specific instructions and/or recommendations for use, storage and handling, including mandatory restrictions proposed as a condition of the authorization applied for

Handling
Instructions for handling, mentioned in the material safety data sheet, are standard precautions for powdered products or products generating fine dust.
In particular:

- Avoid dust dispersion during grinding
- Provide mechanical exhaust or ventilation
- Clean and eliminate dust from equipments regularly
- Use anti-static equipment
- Avoid heat sources and sparks

The product may cause sensitisation by inhalation and skin contact (as any protein-containing product). It may also cause feelings of discomfort.
In particular, wear:

- A mask (paper mask)
- Safety goggles with skin contact
- Gloves
- Wear protective clothes covering skin (discard or wash protective clothes after exposition to dust of the product).

Storage
The product shall be stored at dry conditions in standard silos and kept away from ignition and heat sources

Use in compound feedingstuffs:

* Pigs (for fattening, grower- finisher)
Maximum incorporation rate in the feed: 12% (as is basis)

* Dairy cows (for milk production) & ruminants (in general for meat and milk production as from the beginning from rumination)
Maximum incorporation rate in the feed: 7.3% on dry matter basis (~8% on 'as is' basis) dairy cows

* Salmonids
Maximum incorporation rate in the feed: 13% (or replacement of 20% of fish meal, feedingstuff containing 65% fish meal).

e) Any proposed packaging requirements

Except pelletisation the product will be sold as such in bulk to feed mills. There are no proposed packaging requirements.

f) A proposal for labeling in accordance with Articles 13 and Articles 25 of Regulation (EC) 1829/2003. In the case of GMOs, food and/or feed containing or consisting of GMOs, a proposal for labeling has to be included complying with the requirements of Article 4, B(6) of Regulation (EC) 1830/2003 and Annex IV of Directive 2001/18/EC

a) As feed material

- Feed material
- *(Name)*: Bacterial protein, by-product from the production of L-threonine, produced from genetically modified microorganism
- Nitrogen expressed as crude protein
- Moisture: maximum 12%
- Crude Ash
- Approval number (Regulation (EC) No 183/2005): **α FR 80 021 090**
- Batch number
- Expiry date
- Net quantity
- Name and address of the producer (which is also the person placing the product on the market)

b) Declarations to be made on the label or packaging of compound feeding stuffs

- *The name*: 'Bacterial protein, by-product from the production of L-threonine, produced from genetically modified micro-organism'
- Amount of the product contained in the feedingstuffs.
- Percentage of the total crude protein provided by non-protein nitrogen

As the product will be delivered in bulk to feed mills (delivery by means of tank trucks), the information corresponding to labelling will be provided to customers by means of the commercial documents preceding or accompanying the delivery of the product (taking into account the official language of the country of destination) and the commercial technical sheet corresponding to this product.

g) Unique identifier for the GM microorganism in accordance with Regulation (EC) 65/2004

Not applicable.

Strain AG3139 is used for production in containment only.
The manufacturing process of L-threonine and of the by-product/ dried killed bacterial biomass PT73 (TM) ensures that the final product will not contain viable cells or transferable DNA of the L-threonine producer GM microorganism.

h) If applicable, geographical areas within the EU to which the product is intended to be confined under the terms of the authorization applied for. Any type of environment to which the product is unsuited

The authorization sought by the Applicant for a use as feed material of the product 'PT73 (TM)' concerns the EU market.

Environment to which the product is unsuited: not applicable.

9. Measures suggested by the applicant to take in case of unintended release or misuse as well as measures for disposal and treatment

The product PT73 (TM) does not contain viable cells and no transferable DNA of the L-threonine-producer GM microorganism. Therefore, it is not necessary to take measures, because no unintended release or misuse is expectable.

B. INFORMATION RELATING TO THE GMM

1. Characteristics of the recipient or (when appropriate) parental organism

1.1 Identity

a) Common name Strain *Escherichia coli* K-12, MG1655
b) Strain designation Strain designation is that provided in the literature. The strain is only a laboratory strain used in the case of the applicant, for the construction of the final L-threonine producer microorganism (i.e. strain AG3139).
c) Source of the strain *E. coli* K-12
d) Accession number from a recognized culture collection Available in several culture collections

1.2 Taxonomy

a) Genus *Escherichia*
b) Species *Escherichia coli*
c) Subspecies Not applicable
d) Strain K-12, - MG1655 (mutant of *E. coli* K-12) Confirmation of the taxonomic position of strain MG1655 (and of the final threonine producer: strain AG3139) was made using ribotyping and serotyping.

1.3 Other names

Not applicable.

1.4 Phenotypic and genetic markers

a) Phenotypic and genotypic information relevant to identification, genetic stability and safety

- Single cells, which are Gram-negative rods and not sporulating
- Colony: size around 2 to 3 mm, round, rough and whitish with a clear edge
- Glucuronidase activity
- No evidence of instability of the recipient strain

b) Information on pathogenicity

As a mutant of *E. coli* K-12, the body of knowledge concerning the absence of pathogenicity of *E. coli* K-12 also applies to the strain *E. coli* K-12 MG1655
Confirmation of the taxonomic position of strain MG1655 was made using ribotyping and serotyping
The results of the molecular typing study performed on MG1655 (and the threonine producer: strain AG3139) are described in B.1.11.d of this summary.
Moreover, the genome of strain *E. coli* K-12 MG1655 has been entirely sequenced.

c) Biological properties

Following nutritional and physicochemical demands apply to the development of the *E. coli* K12 strain:

- Facultative anaerobic micro-organism with optimum growth temperature of 37 °C and optimum growth pH between 6.8 and 7.2,
- *E. coli* K12 requires a supply of organic carbon,
- *E. coli* K12 requires a supply of nitrogen.

1.5 Degree of relatedness between recipient and donor(s), when appropriate

Except for one genetic modification, the sequences introduced or modified in the final strain AG3139 are all coming from *E. coli* K-12 genomes or vectors/ transposons developed from *E coli* K-12 strains.

1.6 Description of identification and detection techniques

Ribotyping and serotyping are used as identification and detection techniques. However, it is not considered relevant to develop specific detection techniques for the parental strain MG1655 considering that it is a laboratory strain well described in the literature.

1.7 Sensitivity, reliability and specificity of the detection techniques

This section is not relevant for the recipient strain MG1655, as it is a laboratory strain which has been well described in the literature. The ribotyping and serotyping techniques mentioned in 1.6, with serotyping being based on PCR are sensitive, reliable and specific.

1.8 Source and natural habitat of the recipient microorganism

Not applicable for GM product falling within Group 2 according to the EFSA guidance document for this application.

1.9 Organisms with which transfer of genetic material is known to occur under natural conditions

The recipient strain MG1655 has the following genotype: F^- , $P1^-$, λ^-. This strain does not have conjugative plasmids or self-transmissible plasmids. Therefore, the possibility of natural transfer is expected to be very low.

1.10 Information on the genetic stability of the recipient microorganism

No evidence of instability of the recipient strain.

1.11 Pathogenicity, ecological and physiological traits

a) Classification of hazard according to the current Community legislation

E. coli K12 strains are to be categorized in Group 1 according to Directive 2000/54/EC within the European Union. Microorganisms in this group are biological agents, which are unlikely to cause human disease.

b) Information on the doubling time and of the mode of reproduction

E. coli K-12 has a doubling time of less than one hour. The mode of reproduction is the vegetative form.

c) Information on survival, ability to form spores or other survival structures

E. coli K12 as well as the recipient strain do not produce spores.
The literature reports that *Salmonella*, *Campylobacter*, *Escherichia*, *Shigella*, *Vibrio* species, and species from other genera can exist in a state where they are viable but cannot be cultured by normal microbiological methods. This differentiation of vegetative cells into a dormant ‘’viable but non-culturable’’ (VNC) state was considered as a survival strategy for many non-sporulating species.
Studies performed on an *E. coli* K-12 strain in soil and water show that the *E. coli* cells were disappearing from the non sterile microcosms studied (perhaps consumed by indigenous microorganisms) and raising the question of whether the VNC state would be irrelevant in natural environments. From these elements the conclusion may be drawn that a significant increase of *E. coli* K-12 survival ability through the VNC state is far from being clearly established.

d) Infectivity

- *E. coli* K12 is listed as a non-pathogenic micro-organism.

- Safety has been extensively reviewed and *E. coli* K12 is known as non-toxigenic.

- A molecular typing study performed on MG1655 (and the threonine producer AG3139), based on the detection by PCR of the genes encoding the virulence factors of *E. coli,* showed the absence of these factors in strain MG1655 (and strain AG3139). The host strain MG1655 and the threonine producer AG3139 do not contain genes of virulence factors.

- *E. coli* strains have not been reported to cause allergic reactions.

On the basis of the information available in the literature on the safety of *E. coli* K-12 in general, on the recipient strain MG1655 and the additional studies performed on MG1655 (and the final threonine producer: strain AG3139) the absence of pathogenicity of the recipient strain is considered to be sufficiently proven.

e) Presence of genes that confer antibiotic resistance

The *E. coli* K-12 strain MG1655 has not been reported to present antibiotic resistance.

f) Involvement in environmental processes

Not applicable, because this concerns a strain which is only used at laboratory level.

1.12 Information on indigenous mobile genetic elements

The recipient strain *E. coli* K-12 MG1655 has not been reported to have functional mobile genetic elements such as transposons, prophages, plasmids and sex factors. Like strain *E. coli* K-12, several mobile insertion elements are present in the genome of the recipient strain MG1655.

1.13 Description of its history of use

The recipient strain MG1655 has not been used *per se* and is only an intermediate strain/starting point for the construction of the L-threonine-producing strain AG3139. As regards the general use of *E. coli* K-12 strains see B.1.11.

1.14 History of previous genetic modifications

The recipient strain MG1655, mutant of *E. coli* K-12, was obtained from *E. coli* K-12 wild type by techniques of mutagenesis (by UV radiation and mutagenic agent treatment).

2. Characteristics of the donor organism(s)

Except one genetic modification, the sequences introduced or modified in the final strain TK7 are all coming from *E. coli* K-12 derivative strains. For these *E. coli* K-12 derivative strains the elements of this section are already described in part B.1 of this summary. Below, only the non-confidential characteristics of the other donor organism are described.

2.1 Identity

a) Common name (of the non *E. coli* K-12 donor microorganism) Not applicable as this donor microorganism is used at Ajinomoto laboratory level only.
b) Strain designation (of the non *E. coli* K-12 microorganism) *E. coli* H155
c) Source of the strain Strain used at Ajinomoto laboratory level only.
d) Accession number from a recognized culture collection Not applicable as the strain *E. coli* H155 used at Ajinomoto laboratory level has not been deposited in any culture collection.

2.2 Taxonomy

- Class: Scotobacteria
- Order: Eubacteriales
- Family: Enterobacteriaceae
- Genus: Escherichia
- Species: *Escherichia coli*
- Strain: H155 not clearly defined

2.3 Other names

No other name for the donor microorganism (*E. coli* H155).

2.4 Phenotypic and genetic markers

Certain characteristics of this strain are considered as confidential information. Therefore, no details are provided in the summary.

2.5 Description of identification and detection techniques

It is not relevant to develop specific identification and detection techniques for this donor strain (as it is a laboratory strain). Only the amino acid producing strains are relevant in this respect.

2.6 Sensitivity, reliability and specificity of the detection techniques

This section is not relevant for the donor strain *E. coli* H155 considering that it is a laboratory strain.

2.7 Source and habitat of the organism

This section is not relevant, because the donor organism (*E. coli* H155) is a laboratory strain.

2.8 Pathogenicity traits

Studies carried out on strain *E. coli* H155 have shown the absence of any factor of adhesion, invasion, survival in tissues, cytotonicity or cytotoxicity and of diarrhoeagenic enterotoxins. These results, thus, allow the conclusion that this *E. coli* strain will not be pathogenic to humans or animals.

2.9 Description of its history of use

The applicant is not aware of information about the usage of this donor strain used (*E. coli* H155), beyond its own usage of the strain and the studies it has performed.

3. Description of the genetic modification process

3.1 Characteristics of the vector

This information is considered as confidential information. Therefore, no details are provided in the summary.

3.2 Information relating to the genetic modification

This information is considered as confidential information. Therefore, no details are provided in the

summary.

4. Identification of the conventional counterpart microorganism and its characteristics

Not applicable for GM product falling within Group 2 according to the EFSA guidance document for this application.

5. Information relating to the GMM and comparison of the GMM with its conventional counterpart

5.1 Description of the genetic trait(s) or phenotypic characteristics and any new trait which can be expressed or no longer expressed

As indicated in section B.1, studies using ribotyping and serotyping were performed with the threonine producing strain AG3139 in comparison with the initial specific host strain *E. coli* K-12 MG1655. They confirm that strains AG3139 and MG1655 originate from the same strain *E. coli* K-12.

A study was performed to confirm the antimicrobial susceptibility of the strain AG3139 showed that this one was susceptible to all antibiotics tested.

A significant increase of the metabolic flow towards L-threonine synthesis for the production of L-threonine is observed in strain AG3139, compared with the host strain MG1655.

The other differences between strain AG3139 and the host strain resulting from the construction of the former are considered as confidential information and are, therefore, not included in this summary.

5.2 Structure and amount of any vector and/or donor nucleic acid remaining in the final construction of the modified microorganism

In order to guarantee that none of the antibiotic resistance genes used for intermediate constructions could be present in the genome of strain AG3139, southern hybridization experiments were performed for these antibiotic resistance genes. For all the antibiotic resistance genes tested, no band was observed in the lane corresponding to the DNA of strain AG3139, thus showing the absence of these antibiotic resistance genes in the final construction/strain. These results are in good accordance with the data obtained concerning the research of antimicrobial susceptibility in B.5.1.

The other pieces of information relating to this section are considered as confidential and are, therefore, not included in this summary.

5.3 Stability of the microorganism in terms of genetic traits

To follow the phenotypic stability, the ability to produce threonine is verified by fermentation tests after several generations corresponding to the industrial process using different strains stocks.

A stability study was also performed, at laboratory scale, by analysis of the genomic structure by hybridization after cultivation of the strain AG3139 during 15 to 20 generations, including L-threonine production. Genomic DNA was prepared before and after 15 to 20 generations and digested by restriction enzymes. The DNA fragments were then separated on agarose gel and transferred onto membrane. The same pattern of hybridization was observed before and after culture. This shows the stability of the copies integrated in the genome even after 15 to 20 generations.

Other information relating to this section is considered as confidential information and, therefore, not provided in this summary.

5.4 Rate and level of expression of the new genetic material

Not applicable for GM product falling within Group 2 according to the EFSA guidance document for this application.

5.5 Description of identification and detection techniques

For strain AG3139 two techniques of detection are available:

- The first one is also used as traceability method for PT73 (TM) consisting of the dried killed cells of strain AG3139 and is described in Part V of the application dossier;
- The second one is the monitoring of the integration cassettes in the genome by southern hybridization.

5.6 Information on the ability to transfer genetic material to other organisms

To limit any potential transfer of genetic material to other organisms, the strategy of construction for the strain AG3139 strain was based, among others, on:

- Chromosomal insertion for the genes introduced, rather than using extrachromosomal elements;
- Avoiding that, genes useless for threonine production, such as marker genes of antibioresistance, which could confer a selective advantage to the recipient microorganism, remain at the end of the construction.

Moreover, except for one modification, the only genes transferred in the final strain AG3139 correspond to the over-expression of existing metabolic enzymes for the synthesis/production of amino acids.

Other information relating to this section is considered as confidential information. Therefore, no details are provided in the summary.

5.7 Information on the interaction of the GMM with other organisms

Not applicable for GM product falling within Group 2 according to the EFSA guidance document for this application.

5.8 History of previous releases or uses of the GMM

Not applicable to the GMM used to produce PT73 *E. coli* (THR). This microorganism has not been subject to any release.

5.9 Safety for humans and animals

a) Information on any toxic, allergenic or other harmful effects on human or animal health

Considering that the genetic modifications made resulting in strain AG3139, with one exception, exclusively correspond to the over-expression of existing metabolic enzymes for the synthesis/production of amino acids, this section is not applicable.

A molecular typing study was performed on the initial host strain and on the derived L-threonine-producing strain AG3139 to detect by PCR the genes encoding the virulence factors of *E. coli*. None of the genes encoding the virulence factors investigated were found to be present in the L-threonine-producing strain AG3139.

b) Potential for DNA transfer or any capacity for enhanced gene transfer

To limit any potential transfer of genetic material to other organisms, the strategy of construction for the strain AG3139 strain was based, among others, on:

- Chromosomal insertion for the genes introduced, rather than using extrachromosomal elements;
- Avoiding that, genes useless for threonine production, such as marker genes of antibioresistance, which could confer a selective advantage to the recipient microorganism, remain at the end of the construction.

Moreover, except for one modification, the only genes transferred in the final strain AG3139 correspond to the over-expression of existing metabolic enzymes for the synthesis/production of amino acids.

A part of this section is also answered in section C.4 (risk assessment for DNA transfer).

Other information relating to this section is considered as confidential information. Therefore, no details are provided in the summary.

c) Viability and residence time of the GMM in the alimentary tract

Not applicable to PT73 (TM) deriving from the GMM as this biomass does not contain viable cells of the GMM.

d) Information on any impact of the GMM on the microbiota of the human or animal gastrointestinal tract

Not applicable to PT73 (TM) deriving from the GMM as this biomass does not contain viable cells of the GMM.

5.10 Information on monitoring, control, waste treatment and emergency response plans

This section is not applicable to final products deriving from GMM falling within Group 2 according to the EFSA guidance document of this application.

C. INFORMATION RELATING TO THE GM PRODUCT

'PT73 (TM)', intended to be used as feed material (concentrated source of protein), is a by-product of the manufacturing process of L-threonine by fermentation. It consists of the dried killed cells of the microorganism producing L-threonine (*E. coli* K-12 strain AG3139).
Ajinomoto Eurolysine has already applied, under article 17 of Regulation 1829/2003, for the authorisation of such a threonine-*E. coli* K-12 derived bacterial biomass – 'PT73 *E. coli* (THR)', under the reference EFSA-GMO-FR-2007-44 - however deriving from another *E. coli* K-12 strain (i.e. strain TK7).

Compared with the previous threonine-*E. coli* K-12 derived bacterial biomass 'PT73 *E. coli* (THR)', besides the change of strain (described in section B), the manufacturing processes of L-threonine and of its derived bacterial biomass are basically kept unchanged.
Only minor changes, rather fine-tunings, were made concerning operating conditions for the:

- fermentation phase: adjustments to the needs of strain AG3139,
- separation of the bacterial cells from the inactivated fermentation broth and washing of the 'cell creams' recovered to improve the efficiency of these steps.

Considering that:

- the safety of strain AG3139 being established, no additional risks / hazards are introduced in the manufacturing processes of threonine and of the bacterial biomass because the raw materials and equipments used are not modified, and
- the intrinsic characteristics of the bacterial biomass (accessibility to the digestive enzymes, digestibility, etc.) are not changed even if the content in certain components of the bacterial biomass may vary between PT73 (TM) and PT73 *E. coli* (THR),

the data collected for PT73 *E. coli* (THR) regarding manufacturing process, nutritional value and toxicity are regarded as applicable for the assessment of PT73 (TM).

Nevertheless, additional genotoxicity tests were specifically performed on PT73 (TM) to confirm its safety for animals and consumers of animal derived product. Their results are in accordance with those obtained on PT73 *E. coli* (THR)

For these reasons, different studies performed on PT73 *E. coli* (THR) derived from the corresponding sections of the PT73 *E. coli* (THR) application dossier under reference EFSA-GMO-FR-2007-44 are included in the different sections instead of performing new studies on PT73 (TM) to substantiate the nutritional and toxicological safety of PT73 (TM).

1. Information relating to the production process

PT73 (TM) is a by-product of the manufacturing process of L-threonine by fermentation. L-threonine is produced by fermentation process ('fed-batch fermentation') of a selected strain of *E. coli* K12, which has been modified to produce L-threonine. The fermentation culture medium consists of carbon sources, nitrogen sources, salts, amino acids and vitamins. The production of threonine and, then, PT73 (TM) consists of the following steps (chronological): strain preservation, culturing of seeds, fermentation using the sterilised raw materials of the fermentation culture medium, ammonia and filtrated air. Afterwards the broth is inactivated and subjected to further processing containing the following steps (chronological): recovery and washing of the inactivated cells of the L-threonine producer, decantation and concentration of the bacterial cells, cell drying, pelletisation, cooling, sieving and storage.

2. Information relating to the product purification process

2.1 Technique used to remove microbial cells from the product

Not applicable since the microbial cells are not removed from the product. PT73 (TM), in essence, contains inactivated and denaturated microbial cells.

2.2 Information on the technique used to kill the microbial cells

The micro-organism inactivation procedure has been defined on the basis of bibliographic data on the sensitivity of *E. coli* to heat. As all vegetative cells, *E. coli* shows a high sensitivity to heat treatment, contrary to spore-forming organisms.

The possible presence of viable cells of Strain AG3139 in PT73 (TM) after the inactivation procedure is investigated by detection of bacterial growth of viable cells after plating on *E. coli* specific media adapted to this strain. No viable AG3139 cell was detected in three different production samples/batches, in 1 gram of each sample tested

The efficacy of the inactivation treatment developed by AEL and applied to the 'threonine fermentation broth' to effectively kill the cells of the *E. coli* K-12 strain producing L-threonine had already been confirmed, as described in the dossier for the application for authorisation of PT73 *E. coli* (THR) and confirmed by the French competent authority 'Commission de Génie Biomoléculaire'.

As described for 'PT73 *E. coli* (THR)', besides cultures on specific medium additional studies were performed on the inactivated broth, which were based on the FISH and DVC FISH methods. FISH (fluorescence in situ hybridization) detects viable bacteria and bacteria which died in the last 48h. DVC FISH (direct viable count FISH) allows to identify and detect metabolically active bacteria. All bacteria (viable, viable but non culturable, and dead bacteria) can be detected by DAPI (DiAmidinoPhenylIndole) staining.

The results of these methods were compared and confirmed the efficacy of the inactivation treatment. These results are also considered to be representative for PT73 (TM).

2.3 Information on the process used to purify the product from the microbial growth medium

This section, mainly introduced for 'purified' products, such as amino acids, enzymes, etc., is not really applicable to products such as PT73 (TM).
It may be indicated that the inactivated bacterial cells making up PT73 (TM) are washed during their recovery.

3. Description of the product

3.1 Designation of the product

PT73 (TM) is a dried killed bacterial biomass, a by-product of L-threonine production by fermentation using a genetically modified strain of *E. coli* K12. Like the previous threonine-*E. coli* K-12 derived bacterial biomass PT73 *E. coli* (THR), it is intended to be used as feed material (concentrated source of protein). PT73 *E. coli* (THR) has a complex nature and does not contain viable cells or transferable DNA of the GMM. The proposed commercial name is 'PROT-AEL-T' (standing for 'Protein- Ajinomoto Eurolysine- Threonine')

The product will be delivered in bulk to feed mills. The information corresponding to labelling will be provided to customers by means of the commercial documents preceding or accompanying the delivery of PT73 (TM) and the commercial technical sheet corresponding to this product.

3.2 Intended use and mode of action

The product is intended to be used as a feed material supplying protein (concentrated source of protein) in compound feeding stuffs for pigs (grower-finisher), fish (salmonids), ruminants (in particular dairy cow).

3.3 Composition

-A compositional analysis was performed for several parameters to determine its main and minor components (also in view of assessing its nutritive value) and the occurrence of potential contaminants. Some analyses of micro-components (such as vitamins) and potential contaminants (dioxins, dioxin-like PCBs, PAHs, residues of pesticides) previously performed on PT73 *E. coli* (THR) in the framework of the application under the reference EFSA-GMO-FR-2007-44 were not carried out again on PT73 (TM) as they were considered applicable to the latter as well. In this case, the values found for PT73 E. coli (THR) were used for PT73 (TM). Compositional analysis was performed on:

* Nitrogen components (total and free amino acids, ammonium N, amide N, urea N, biogenic amines, nitrates and nitrites, nucleic acids)
* Total lipids, fatty acids
* Carbohydrate fraction
* Organic acids
* Inorganic components
* Vitamins
* Potential contaminants: heavy metals, organochlorine and organophosphorus pesticides, dioxins, PCBs, polyaromatic hydrocarbons

Compared with the threonine biomass PT73 *E. coli* (THR) obtained using the strain TK7 (it may be observed that the threonine biomass PT73 (TM) shows a higher total nitrogen and, correspondingly, crude protein content (on a Dry Matter basis and on an 'as is' basis) than the threonine biomass PT73 *E. coli* (THR), in spite of a somewhat lower (by 0.2 to 0.3%) content in ammonium. The former also shows a lower crude ash and, more specifically, lower sulphates content than the latter.

This can be attributed to more efficient separation of the bacterial cells from the inactivated fermentation broth and better washing of the cell creams which was one of the objectives looked for during the industrial trial for producing threonine using the strain *E. coli* K-12 AG3139.

The increase in crude protein content and reduction of the levels of sulphates and ammonium in the threonine biomass PT73 (TM) (strain AG3139) compared with the threonine biomass PT73 *E. coli* (THR) (strain TK7) is considered to favorably influence the composition of the threonine biomass and of its nutritive value.

3.4 Physical properties

The results obtained with PT73 *E.coli* (THR) are applicable to PT73 (TM) as these characteristics mainly result from the very nature of the product (*E. coli* K-12 derived bacterial biomass) and of its manufacturing process (kept basically unchanged), such as drying conditions, pelletisation conditions..

PT73 (TM) is a brown solid product with a bulk density of 0.638 kg/L and a pH of 4.0 (in 10 % w/v suspension).

- Electrostatic properties (mJ): 810 < MIE[1] < 1200
- Auto-ignition: 520 °C
- Thermoanalysis: 219 °C (Classified as 'among most reactive dusts')
- Explosivity:
  Pmax: 6.7 bar
  MRPmax: 362 bar/s
  Kst: 98 bar
  Explosion class: St1

3.5 Technological properties

The results obtained with the threonine-*E. coli* K-12 derived bacterial biomass 'PT73 *E.coli* (THR)' are applicable to PT73 (TM) as these characteristics mainly result from the very nature of the product (*E. coli* K-12 derived bacterial biomass) and of its manufacturing process (kept basically unchanged), such as drying conditions, pelletisation conditions.

It is reminded that a large quantity of PT73 *E. coli* (THR) was produced for carrying all the studies necessary for the corresponding application dossier. To minimize any risk of degradation, especially microbial degradation, of this quantity of PT73 *E. coli* (THR) until parts were taken to prepare the various experimental diets for the studies to evaluate its safety and nutritive value or for carrying out stability studies, it was stored in refrigerated conditions at 2-3°C with 70% humidity in big bags of about 1 ton each. This quantity was subject to regular monitoring and microbiological analyses over a total period of 23 months. PT73 *E.coli* (THR) was stable from a microbiological perspective during the storage period of 23 months at 2-3 °C. Therefore, the chemical composition and the nutritive value of the product were not altered due to the activity of microorganisms during this storage.

The effects of different climatic conditions - combinations of different temperatures and relative humidity (RH) - on the behaviour and stability of the threonine-*E. coli* K-12 derived bacterial biomass PT73 *E. coli* (THR), as such, or of compound feedingstuffs containing it (compound feedingstuffs prepared to evaluate its nutritive value) were investigated:
- PT73 *E. coli* (THR) was chemically and physically stable during 12 months storage at 5 different climatic

conditions, covering a wide range of moderate and subtropical conditions. PT73 *E. coli* (THR) demonstrated a good microbiological quality during the storage period and can, therefore, be considered as microbiologically safe feed material.
- Pig feeds and dairy concentrates containing max. 20% PT73 *E. coli* (THR) were chemically stable during 6 months storage at 3 different climatic conditions, covering a realistic range of moderate and subtropical conditions. Furthermore, they did not contain pathogenic microorganisms at hazardous levels.

4. Assessment of the presence of recombinant DNA and of the potential risk of gene transfer

In this section it is shown that the dried killed bacterial biomass – PT73 (TM) – obtained using the *E. coli* K-12 strain AG3139 and the manufacturing process described in this dossier neither consists of, nor contains GMOs, within the meaning of the definitions of 'GMO' and 'organism' provided by Directive 2001/18/EC (on the deliberate release of genetically modified organisms into the environment).

Based on the definition of "organism" provided by Directive 2001/18/EEC (*italics added)*: "any biological entity capable of replication or of transferring genetic material *(to other organism)*", the evaluation of PT73 (TM) was articulated around the:

- Absence of viable cells of the strain AG3139 in the product. The determination of viable cells of AG3139 is based on the use of a specific culture medium.
- Absence of potentially transferable (recombinant) DNA in the product.

No viable cells of strain AG3139 was detected in each of three samples of PT73 (TM) tested.

Agarose gel analysis showed extensive genomic DNA degradation in PT73 (TM).

Measures by conventional and Real Time PCR were performed, with sets of primers/primers-probe targeting specific DNA sequences of the AG3139 genome and used to amplify DNA fragments of 454bp (or more) and 84 bp (or more) for the former and the latter respectively. With conventional PCR, amplification of the DNA fragment of 0.454kb was detected in PT73 (TM), however only in very low quantity and close to the detection threshold (less than 0.001ppm expressed as genomic DNA quantity). By Real Time PCR, the DNA fragment of 84pb was detected, with a quantity of DNA fragments (84bp) significantly higher (by 20 000 times) than the one detected by the conventional PCR method (454bp).
These results confirm that the AG3139 genomic DNA was degraded extensively during the inactivation procedure.

Complementary studies based on the transformation method in laboratory conditions, with an *E. coli* K-12 strain specifically developed for an auxotrophy (for cysteine), were performed to evaluate the potential risk of gene transfer.
The transformation efficiency was determined to be around 8 $x10^4$ transformants per microgram of AG3139 DNA. No transformant prototrophic for cysteine was detected with the equivalent of 100mg of PT73 (TM) (20 transformation experiments, each carried out with the extracts corresponding to 5mg of PT73 (TM)).

General conclusion
In summary, PT73 (TM) contains recombinant DNA fragments. However, these are small size fragments and PT73 (TM) does not contain (or in trace form) DNA fragments with a size corresponding to that of one of the genes inserted in the genome of strain AG3139. Therefore, gene transfer from PT73 (TM) is considered very unlikely. This is in accordance with the results obtained in the transformation experiments.

5. Comparison of the GM product with its conventional counterpart

This section is not applicable to PT73 (TM), intended to be placed on the market as feed material, because no biomass resulting from the production of threonine using a conventional strain of *E. coli* K-12 has been previously manufactured and placed on the market. A comparative risk assessment with a conventional counterpart is thus not possible. A specific risk assessment was therefore carried out. The data previously collected for the threonine-*E. coli* K-12 derived bacterial biomass PT73 *E. coli* (THR) were largely used for the risk assessment of PT73 (TM).

6. Considerations for human health and animal health of the GM product

6.1 Toxicology

As already mentioned, between the threonine-*E. coli* K-12 derived bacterial biomasses PT73 (TM) and PT73 *E. coli* (THR), subject of a previous application for authorisation, only the following changes were made:

- Use of the threonine producer strain *E. coli* K-12 AG3139 (for the former) instead of *E. coli* K-12 TK7 (for the latter),
- Fine-tunings of the operating conditions for the separation of the cells of the microorganism from the fermentation broth and for the washing of the cell creams recovered.

The rest of the manufacturing process downstream the fermentation phase is kept unchanged.

The information supplied on the genetic modifications applied to the recipient strain to result in the threonine producing strain AG3139 on the one hand, and the studies performed on the recipient, donor and final strains on the other hand allow establishing the safety of strain AG3139. PT73 (TM), like PT73 *E. coli* (THR), does not contain any viable organisms, or transferable DNA from the producer strain.
As previously indicated, the manufacturing processes of L-threonine and of its derived bacterial biomass downstream the fermentation phase are basically kept unchanged. No additional risks / hazards are introduced in the manufacturing processes of threonine and of the bacterial biomass because the raw materials and equipments used are not modified.
Therefore, it is considered that the data collected for PT73 *E. coli* (THR) can largely be used for the risk assessment of PT73 (TM). In this summary section, the data for PT73 *E. coli* (THR) and PT73 (TM) are considered interchangeable.

6.1.5.1 Acute toxicity studies

- Acute oral toxicity of the threonine-*E. coli* K-12 derived bacterial biomass PT73 *E. coli* (THR) in rats: LD_{50} > 2000 mg/kg body weight. PT73 *E. coli* (THR) is considered not harmful when ingested. This value is considered applicable to PT73 (TM) as well.

- Acute inhalation toxicity of PT73 *E. coli* (THR) in rats: LD_{50} > 5.25 g/m^3. PT73 *E. coli* (THR) is not harmful when inhaled. This value is considered applicable to PT73 (TM) as well.

- PT73 *E. coli* (THR) is not irritating to the skin and to the eyes. These results for PT73 *E. coli* (THR) are considered applicable to PT73 (TM) as well.

- As any protein containing material, the threonine-*E. coli* K-12 derived bacterial biomass, PT73 *E. coli* (THR) or PT73 (TM) is a potential sensitizer to the skin and by inhalation. As it will be delivered in bulk,

information corresponding to the risk phrases R42/43 ('May cause sensitization by inhalation and skin contact') according to Directive 2001/59/EC[1] will be provided through the product's MSDS and in the document accompanying the delivery.

Overall, it is concluded that the threonine-*E. coli* K-12 derived bacterial biomass (PT73 *E. coli* (THR) or PT73 (TM)) has a low acute toxicity. The product may be a sensitizer (risk phrase R42/43).

6.1.5.2 Subchronic and genetic toxicology studies

- Bacterial Reverse Mutation (Ames) test, chromosome aberrations testing in mammalian cells and gene mutation testing in mammalian cells were carried out on the threonine-*E. coli* K-12 derived bacterial biomass PT73 (TM). PT73 (TM) is not mutagenic. These results with PT73 (TM) are in line with those obtained with PT73 *E. coli* (THR) for the same tests.

- In a 13-week oral toxicity study in rats, the threonine-*E. coli* K-12 bacterial biomass PT73 *E. coli* (THR) was tolerated without obvious signs of toxicity at dietary levels up to 20% (equivalent to ca 10 g/kg bw/d). These results are considered to be applicable to PT73 (TM) as well.

- No effects on reproduction are expected on the basis of the reproduction parameters, which were found to be normal in the subchronic feeding study in rats.

- The results of a developmental toxicity study in the rat with the threonine-*E. coli* K-12 bacterial biomass PT73 *E. coli* (THR) indicated that no effects on development are to be expected from feeding the threonine bacterial biomass to pregnant animals up to dietary levels of 20%. These results are considered to be applicable to PT73 (TM) as well.

6.1.5.3 Target animals

- Pigs can tolerate feed supplemented with up to 10% of the threonine-*E. coli* K-12 bacterial biomass PT73 *E. coli* (THR) without effects on the zootechnical performance or health of pigs. These results are considered to be applicable to PT73 (TM) as well.

- No effects on fertility or reproduction in the target animals are to be expected from the intended use of the threonine bacterial biomass in animal feed on the basis of the fertility and fecundity parameters in experimental animal studies performed.

- No effects on microflora in the gastrointestinal tract, colonisation of pathogens in the GI tract, or increased antibiotic resistance are to be expected from the intended use of the threonine-*E. coli* K-12-derived bacterial biomass, PT73 *E. coli* (THR) or PT73 (TM), in animal feed.

- No residues from heavy metals, pesticides, PAHs, PCBs, dioxins, and mycotoxins originating from the raw materials used in the manufacturing process of the threonine bacterial biomass or that may be formed during this one, are expected in edible commodities or excreta of animals fed this by-product.

Conclusions

It is noted that the threonine-*E. coli* K-12-derived bacterial biomass is a complex mixture of components for

[1] Commission Directive 2001/59/EC of 6 August 2001 adapting to technical progress for the 28th time Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances (Official Journal of the E.U., L225, p.1, 21-8-2001)

which the following considerations apply:

- the components, other than some of the proteins of this *E. coli* K-12 bacterial biomass, are for most usual components/ nutrients present in feed materials (of which metabolism is known),
- the microbial strains (AG3139) producing L-threonine, the dried killed cells of which make up the threonine bacterial biomass are well identified and characterised (do not produce toxins),
- the product does not contain contaminants of toxicological concern at the levels present.

Considering these aspects it was concluded that studies on the metabolism of the threonine-*E. coli* K-12-derived bacterial biomass, here PT73 (TM), were not considered of additional value as not providing additional information.

Overall, it is concluded that, based on studies performed on target and experimental animals as well as on supplemental information and toxicological considerations, the intended use of the threonine-*E. coli* K-12-derived bacterial biomass, PT73 (TM) in animal feed is not expected to result in undesirable biological consequences for target animals or the environment. Pigs can tolerate a maximum incorporation rate of 10% of the threonine bacterial biomass (based on the study performed with PT73 *E. coli* (THR)) in the daily ration. Workers handling the product are advised to take protective measures, which are described in the MSDS (see appendices).

6.2 Risk assessment of newly expressed proteins

The proteins expressed, as a result of the construction of the strain *E. coli* K-12 AG3139 producing L-threonine, are proteins/enzymes of the general metabolism of *E. coli*/*E. coli* K-12 or of the metabolic pathways leading to threonine production and promoting it. It may also be noted that the strain *E. coli* K-12 AG3139 is devoid of any marker gene of antibiotic resistance, as confirmed by different studies, and, thus, of the corresponding expression protein.
On this basis, proteins, which are part of the threonine bacterial biomass, are not considered to be of health relevance.

6.3 Testing of new constituents other than proteins

PT73 (TM) is not known to contain new constituents.

6.4 Information on natural food and feed constituents

PT73 (TM) is a complex product and its constituents (other than some proteins) are found in a number of other feed materials. As a new feed material, without any conventional counterpart to which it could be compared to, even at least partially, PT73 (TM) was assessed as such regarding its safety (section C.6.1. of the dossier) taking into account results from studies performed on PT73 (TM) itself or on PT73 *E. coli* (THR).

6.5 Testing of the whole GM product

The following studies were performed on the whole threonine-*E. coli* K-12–derived bacterial biomass:
- acute toxicity studies [on PT73 *E. coli* (THR)]
- (sub)chronic and developmental toxicity studies [on PT73 *E. coli* (THR)]
- genetic toxicology testing [on both PT73 (TM) and PT73 *E. coli* (THR)]
- studies on target species: cows, pigs and fish [with PT73 *E. coli* (THR)]
  * tolerance studies
  * performance studies
  * digestibility studies

The results are presented in C.6.1 and C.6.9 of this summary.

6.6 Allergenicity

As PT73 (TM) is intended for use in feed, it is noted that regarding animal health, allergenicity is not an issue that needs to be addressed specifically.

6.7 Assessment of allergenicity of newly expressed protein

According to the EFSA Guidance document, Section III. C.6.8 'Regarding animal health, allergenicity is not a significant issue that needs to be addressed specifically'.
Therefore, although PT73 (TM) may contain newly expressed proteins as a result of the construction of L-threonine producer strain AG3139, this section was not specifically addressed.

6.8 Assessment of allergenicity of the whole GM product

As PT73 (TM) is intended for use in feed, it is noted that regarding animal health, allergenicity is not an issue that needs to be addressed specifically.

6.9 Nutritional assessment of GM feed

Threonine-*E. coli* K-12-derived bacterial biomasses can be described as biomasses with a high crude protein content: (813 to 876 g/kg DM for PT73 (TM) and 775 g/kg DM for PT73 *E. coli* (THR)) and, thus, as concentrated sources of protein. Approximately 6.3 to 7 % of the nitrogen is present in the form of ammonium-nitrogen in PT73 (TM) (9.5% in the case of PT73 *E. coli* (THR)). The remaining part of the nitrogen-containing fraction consists mainly of protein and amino acids (see section C.3.3).

The *in vitro* digestibility study (performed on both PT73 (TM) and PT73 *E. coli* (THR)) and *in vitro* measures on PT73 (TM), in combination with the digestibility in pigs and sheep and performance studies in pigs, carried out with PT73 *E. coli* (THR), indicate that (threonine-)*E. coli* K-12-derived bacterial biomasses,

of which PT73 (TM), are suitable protein sources in the diet for pigs (monogastrics), ruminants and fish.

The optimal nutritional level of PT73 (TM) for pigs, based on the results obtained with PT73 *E. coli* (THR), is up to a level of 120 g/kg feed (as is basis). The overall eating quality of meat from pigs fed with a diet containing the threonine bacterial biomass at this level is not found to be different from the control group fed a standard diet without threonine bacterial biomass.

Tolerance and performance studies on dairy cows were not performed with PT73 *E. coli* (THR). However, when comparing PT73 *E. coli* (THR) to another *E. coli* K-12 derived bacterial biomass: PL73 *E. coli* (LYS) deriving from the production of L-lysine, the conclusion can be drawn that threonine-*E. coli* K-12-derived bacterial biomasses - PT73 *E. coli* (THR), and PT73 (TM)- are a source of protein, which is at least as suitable as the biomass PL73 *E. coli* (LYS) in the diet for dairy cows/ ruminants. Its optimal nutritional level of incorporation in the rations for ruminants is up to a level of 73 g/kg feed on a Dry Matter basis (~8% on 'as is' basis).

The digestibility and growth studies in fish (rainbow trout), carried out with PT73 *E. coli* (THR), indicate that threonine-*E. coli* K-12 derived bacterial biomasses, of which PT73 (TM), are a suitable source of protein that can be substituted to fishmeal in feedingstuffs for salmonids (trout). In a fish feed containing 65% fish meal, substitution of up to 20% of the fishmeal by PT73 *E. coli* (THR) did not alter growth performances of the trout nor the animal characteristics.

6.10 Post-market monitoring of GM products

The applicant has the opinion that no post marketing monitoring of PT73 (TM) is necessary for the following reasons;

- As shown in section C.4, this product does not contain viable cells nor transferable DNA of the threonine-producing GMM (strain AG3139).
- The product is intended to be placed on the market as feed material only.
- A somewhat comparable dried killed bacterial biomass resulting from the production of lysine using *Corynebacterium glutamicum/Brevibacterium lactofermentum*[2] has been placed on the market as feed material since 1976 as a product obtained using conventional strains of *B. lactofermentum*, and since January 1998 as a product obtained using GM strains of *B. lactofermentum* (*) without any report of whatsoever adverse effects.

(*): notified as 'existing product' according to article 20 (1) of Regulation (EC) 1829/2003 and for which an application for renewal of authorization - dossier under the reference EFSA-GMO-RX-PL73 – has been remitted to Authorities).

[2]These are two names for the same species: *Brevibacterium lactofermentum* has been re-classified as *Corynbacterium glutamicum*