Journal of Invertebrate Pathology 106 (2011) 386–393

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Identification of *Enterococcus mundtii* as a pathogenic agent involved in the "flacherie" disease in *Bombyx mori* L. larvae reared on artificial diet

Silvia Cappellozza [a,*], Alessio Saviane [a,1], Gianluca Tettamanti [b,2], Marta Squadrin [c,3], Elena Vendramin [c,3], Paolo Paolucci [d,4], Eleonora Franzetti [b,2], Andrea Squartini [c,3]

[a] CRA – Apiculture and Sericulture Research Unit, Padua seat, via Eulero, 6a, 35143 Padova, Italy
[b] Department of Biotechnology and Molecular Sciences, University of Insubria, via J.H. Dunant, 3, 21100 Varese, Italy
[c] Department of Agricultural Biotechnologies, University of Padova, via Romea, 16, 35020 Legnaro (Padova), Italy
[d] Department of Environmental Agronomy and Crop Production, University of Padua, viale dell'Universita', 16, 35020 Legnaro (Padova), Italy

ARTICLE INFO

Article history:
Received 8 September 2010
Accepted 9 December 2010
Available online 15 December 2010

Keywords:
Acridine
ARDRA
Artificial diet
Bacterial disease
Bombyx mori
Chloramphenicol
DAPI
Enterococcus mundtii
Germfree rearing
Mulberry silkworm
Pyronin
TEM

ABSTRACT

Enterococcus mundtii was shown to be directly correlated with flacherie disease of the silkworm larvae reared on artificial diet supplemented with chloramphenicol. Its identification was carried out by means of light and electron microscopy and nucleotide sequencing of 16S gene. The bacterium is capable of rapidly multiplying in the silkworm gut and of invading other body tissues, as demonstrated by deliberate infection of germfree larvae and by subsequent TEM observations. *E. mundtii* can endure alkaline pH of the silkworm gut and it has been proved to adapt *in vitro* to commonly applied doses of chloramphenicol, whose use can further contribute to reduce competition by other bacteria in *Bombyx mori* alimentary canal.

The modality of transmission of the infection to the larvae was among the objectives of the present research. Since contamination of the progeny by mother moths can be avoided through routine egg shell disinfection, a trans-ovarian vertical transmission can be ruled out. On the other hand the bacterium was for the first time identified on mulberry leaves, and therefore artificial diet based on leaf powder could be a source of infection. We showed that while microwaved diet could contain live *E. mundtii* cells, the autoclaved diet is safe in this respect. Being *E. mundtii* also part of the human-associated microbiota, and since *B. mori* is totally domestic species, a possible role of man in its epidemiology can be postulated.

1. Introduction

Rapid advances in biotechnology and realization of transgenic silkworms to create new materials for pharmaceutical or bio-medical applications have highlighted the importance of artificial diets for silkworm larvae. Traditional rearing on mulberry leaves is largely dependent on natural environmental conditions and demanding extensive workers' labor, it does not warrant efficient and predictable production.

In mass-rearing of silkworms on artificial diet, diseases from bacterial infections are frequent causes of total loss of cocoon harvest.

Two major strategies have been developed so far; the first involves rearing under germfree conditions, and the second, supplementation of artificial diet with antibiotics. For the latter, one of the most used antibiotics which can be added to artificial diet is chloramphenicol; the chemical has qualities of a good candidate as an additive for the diet: (1) it is not inactivated in the silkworm gut, despite very alkaline pH of the digestive fluid; (2) it is able to stand high temperatures during autoclave sterilization; (3) it is active on wide groups of bacteria (Gram positive and Gram negative) (Kodama, 2001). For these reasons, it has been used for many years in sericulture, both in rearings on the leaves and on the diet to counteract epidemic outbreaks.

The CRA – Unit of Research of Apiculture and Sericulture, Padua, Italy (CRA-API) is one of the most important silkworm germplasm banks in Europe, comprising around 200 *Bombyx mori* strains. Every year part of rearing for strain reproduction is performed with locally-produced artificial diet (CRA-API's patent) (Cappellozza et al., 2005). Furthermore, CRA-API supplies artificial diet to many European laboratories dealing with the silkworm as experimental tool. After repeated encountering of flabby larvae in artificial diet rearing, using CRA-API's diet containing chloramphenicol, which

* Corresponding author. Tel.: +39 049 620205; fax: +39 049 623119.
E-mail addresses: silvia.cappellozza@entecra.it (S. Cappellozza), gianluca.tettamanti@uninsubria.it (G. Tettamanti), paolo.paolucci@unipd.it (P. Paolucci), squart@unipd.it (A. Squartini).
[1] Tel.: +39 049 620205; fax: +39 049 623119.
[2] Tel.: +39 0332 421312; fax: +39 0332 421300.
[3] Tel.: +39 049 8272923; fax: +39 049 8272929.
[4] Tel.: +39 049 827 2809; fax: +39 049 827 2839.

0022-2011/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2010.12.007

occurred both in Padua and in other European labs, we focused our efforts on isolation of possible etiological agent for the so-called "flacherie" disease. Larvae showing symptoms of retarded growth, cessation of feeding, flaccidity, loss of body lustre, sluggishness and dysentery can be affected by bacteria or viruses (Aizawa et al., 1964; Tanada and Kaya, 1993; Lysenko, 1958) or even by two microbial agents (Manimegalai, 2009; Deseö Kovács and Rovesti, 1992).

The research in the current paper aimed at identifying unambiguously the cause of the pathology and its epidemiological boundary conditions to establish sound remedies.

2. Material and methods

2.1. . Experimental animals

Polyhybrid larvae (126 × 57) (70 × 79) produced at CRA-API were used. Eggs were surface sterilized following the method of Matsubara et al. (1988) for germfree rearing. Larvae were fed from egg hatching on artificial diet or mulberry leaves, *Morus alba*, cultivated varieties Florio and Morettiana. Larvae were sacrificed for gut analysis between 72 and 120 h from the beginning of the last instar. Before sacrificing larvae, a disinfection test on the surface of larval body was carried out: larvae were anaesthetized with ethyl acetate, dipped in 95% ethanol for 10 s, then in 5% sodium hypochlorite for 1 min and rinsed four times in distilled water.

2.2. . Artificial diet

Composition of artificial diet is described in Cappellozza et al. (2005) and chloramphenicol (Sigma–Aldrich) was added before autoclaving (117 °C for 40 min) or microwave cooking (10 min at 850 W) at the dosage of 0.1 μg/g. Diet was prepared in a thin layer of around 2.5 cm of thickness, in a plastic container covered with a plastic wrap.

2.3. . Bacteria isolation from silkworm gut

Extraction was carried out from 5th instar healthy and diseased larvae reared under non-germfree conditions, from 5th instar healthy and purposely infected larvae reared under germfree conditions (Sumida and Ueda, 2007), and from 5th instar larvae reared on mulberry leaves. For extraction from the intestine, larvae were placed on a polystyrene layer, and fixed at both ends with two pins. The support and cutting instruments were previously disinfected by immersion in 95% ethanol. The larvae were cut with scissors making a cut along the back and pulling with tweezers the digestive tract that was shredded and deposited in an eppendorf tube containing 200 μl of sterile saline solution. For each sample serial dilutions of up to 10^{-6} were made, and for each dilution two plates of Plate Count Agar (PCA Difco) were seeded by plating 40 μl of suspension. Plates were sealed with parafilm and incubated at 22 °C.

2.4. . Fluorescence microscopy analysis of bacterial isolates

Aliquots of 20 μl of suspensions from gut isolation obtained as described above were placed on glass slides. Slides were fixed on the flame, and stained with 0.1% acridine orange in deionized water for 1 min, rinsed four times with distilled water and air dried. The slides were observed with epifluorescence Olympus microscope with a 100× objective and images were acquired with Olympus Camedia digital camera.

2.5. . Bacterial species identification with molecular methods

To characterize isolated bacteria taxonomically, cell lysis and DNA amplification were performed. Single colonies were picked from plates and resuspended in 50 μl of lysis solution (0.25% sodium dodecylsulphate and 0.05 M NaOH) in a 0.5 ml eppendorf tube. Tubes were vortexed for 1 min, heated at 94 °C for 20 min. Samples were centrifuged for 10 min at 13,200*g* and 10 μl of supernatant were withdrawn and added to 90 μl sterile water. For 16S rDNA PCR amplification the following reagents were used per reaction: 1 μl of lysate template, 12.3 μl of milliQ sterile water, 2.5 μl 10× buffer; 4 μl of dNTPs at a concentration of 1.25 mM each, 2.5 μl of primer 63F (Marchesi et al.,1998) and 2.5 μl of primer 1389R (Osborn et al., 2000) both at a concentration 2.5 μM; 0.2 μl of Taq enzyme at a concentration of 5 units/μl.

PCR was achieved in I-Cycler BioRad thermal cycler, set with the following program: DNA denaturation at 94 °C for 2 min; annealing at 54 °C for 30 s; extension at 72 °C for 2 min and 30 s, repeated 35 cycles, and a final extension at 72 °C for 5 min. Amplicon DNA was checked by 1% agarose gel electrophoresis and photographed with Kodak digital apparatus EDAS290. The same method applied to the colonies was also used for lysis and amplification directly from suspension-dilutions of the intestinal suspensions from *B. mori* or from artificial diet.

An ARDRA (Amplified Ribosomal DNA Restriction Analysis) was used to pre-screen taxonomically the amplicons digesting a 5 μl aliquot of amplified DNA with the *Hin*fI enzyme at 37 °C for 2 h. The cut DNA fragments were separated by 1.5% agarose gel. Dideoxynucleotide sequencing of 16S rDNA amplicons was carried out in ABI Prism (Applied Biosystems) automatic sequencer using separately the forward or reverse of the above primers. Chromatograms were subjected to BLAST analysis using the online platform NCBI (http://www.ncbi.nlm.nih.gov/).

2.6. Histochemistry

Midgut of diseased larvae underwent a specific histochemical staining for flacherie virus (Veda et al., 1997). Ten larvae at the fifth day of the 5th instar were analyzed. Anesthetized larvae were cut dorsally, and the midgut was dissected and cut in small pieces. For whole mount staining, specimens were incubated with the following solution: 0.6% pyronin Y, 0.3% methyl green, 20% chloroform (v/v) in phosphate buffer solution (0.05 M, pH 4.0). Although in preliminary tests different times of incubations with the staining solution were evaluated (15–90 min), no difference was seen among the processed samples, therefore 60 min-incubation time was chosen for subsequent experiments. Samples were mounted in Citifluor, covered with a coverslip, and examined with an Olympus BH2 microscope. Images were acquired with a Nikon DS-5M-L1 digital camera system.

2.7. DAPI staining

Midgut samples were obtained as described in Section 2.6, immediately embedded in poly-freeze cryostat embedding medium (Polyscience), and stored in liquid nitrogen until use. Cryosections (8 μm thick) were rinsed with PBS for 5 min and subsequently incubated for 20 min with 4,6-diamidino-2-phenylindole (DAPI; 100 ng/ml) to detect DNA. Coverslips were mounted with Citifluor, and slides were examined with an Olympus BH2 microscope.

2.8. Light microscopy and transmission electron microscopy (TEM)

Larval midgut. Midguts of ten diseased larvae were processed for TEM analysis according to Tettamanti et al. (2007). Briefly, after