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Use of metabolic and molecular methods for the identification of a *Bacillus* strain isolated from paper affected by foxing

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Summary

Foxing of paper is a deterioration phenomenon occurring in the form of brown-yellowish spots, the abiotic and/or biotic causes of which are not yet completely understood. Nevertheless, microbiological infection has been recognized that may contribute to paper damage and therefore it becomes important to know the taxonomic position and the degradative activity of the potential infectious biological agents which mostly are fungi, but also bacteria and yeasts. A cellulolytic bacterial strain isolated from a foxed paper sample exhibited morphological and physiological characteristics of the *Bacillus* genus. To study its taxonomic position, different identification methods were used: the Biolog system, the direct amplified polymorphic DNA-polymerase chain reaction analysis (DAPD-PCR) and the partial sequencing of the 16S rDNA gene. Biolog system and partial sequencing of 16S rDNA gene assigned the strain to the *Paenibacillus polymyxa* species. DAPD-PCR analysis indicated a high similarity with *Bacillus circulans*, by comparing the isolated strain with some closely related *Bacillus* species.

Introduction

Old paper frequently develops irregular, yellow-ish-brown and sometimes fluorescent spots which constitute a condition known as ''foxing''. It is a common phenomenon that can arise as a result of biological, chemical and physical factors, the possible causes of which are still matter of debate. However, it has been recognized that a microbiological infection may initiate this damage to paper and many fungal species have been isolated and characterized (Meynell and Newsam, 1978; Arai,

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1984, 2000; Florian and Manning, 2000; Montemartini Corte et al., 2003). Several bacteria have been also isolated from foxed papers (Gallo and Pasquariello, 1989; Lippi et al., 1995; Montemartini Corte et al., 2003), but to our knowledge no study concerning their taxonomical identification or paper degrading activity has been published. On the other hand, an important step in studying the microbial role in paper deterioration processes is to know the taxonomic position of the potential biological agent responsible or co-responsible of the infection, as the isolated bacterial species may represent an important nutritional contribution to fungal growth on paper and can often display other modifications of this cellulosic substrate.

In a previous work (Lippi et al., 1995), we isolated from paper affected by foxing an actively growing bacterial strain that also exhibited high endo- and exo-1,4 β-glucanase activities (Lippi et al., 2001). This strain showed some morphological and physiological characteristics as a *Bacillus* sp., genus widely distributed in different environments, among which the pulp and paper industry. As sterile conditions inside a paper mill are impossible, many bacterial species are adapted to this environment also because the paper manufacturing process is an open system which provides good conditions (nutrients, suitable pH and temperature) for microbial growth (Väisänen et al., 1998; Oppong et al., 2003; Suihko and Stackebrandt, 2003; Suihko et al., 2004).

The taxonomic study of environmental *Bacillus* isolates has been reported to face some difficulties, when available general identification methods are used: biochemical (i.e. FAME) or metabolic (i.e. API or BIOLOG systems) identification tests are not always reliable tools for the identification of bacilli. However, the Biolog system (Garland and Mills, 1991), which has been developed to identify bacteria on the basis of differential utilization of a wide range of C-substrates, may represent a useful and rapid tool for assessment of the metabolic profile of bacteria even though this system offers only a preliminary taxonomic screening test for *Bacillus* species (Nolla Pires and Seldin, 1997).

The shortcomings of phenotypically based identification methods have led to the development of molecular alternatives based on the microbial genotype or DNA sequence. The utilization of different metabolic and molecular methods is highly recommended to support an unambiguous identification of *Bacillus* strains isolated from different environments (Baillie et al., 1995; Väisänen et al., 1998; Guinebretiere et al., 2001; Suihko and Stackebrandt, 2003; Wu et al., 2006).

The amplification of anonymous segments of DNA using short arbitrarily constructed primers (Welsh and McClelland, 1990; Williams et al., 1990) provides fingerprints which can be used for inter- and intra-specific strain differentiation. Amplification is generally obtained from extracted genomic DNA (Random Amplified Polymorphic DNA-polymerase chain reaction analysis: RAPD-PCR), but it is possible to amplify DNA directly from cells (Direct Amplified Polymorphic DNA-polymerase chain reaction analysis: DAPD-PCR) of different bacteria (Newman and Van Taoi, 1990; Harrison et al., 1992) and this technique has been also applied to some *Bacillus* species (Brousseau et al., 1993; Stephan et al., 1994; Vollù et al., 2003).

A very accurate method routinely used in identifying bacterial species is the 16S rDNA gene sequence determination. Several authors have emphasized the usefulness of this molecular method for a rapid identification of *Bacillus* and related species (Goto et al., 2000, 2002; Guinebretiere et al., 2001; Wu et al., 2006) even if the 16S rDNA gene sequencing has shown that the genus *Bacillus* is phylogenetically very heterogeneous (Ash et al., 1993).

The aim of the present work was to achieve a correct taxonomy of the cellulolytic *Bacillus* strain isolated from foxed paper (Lippi et al., 1995, 2001) by utilizing some of the abovementioned metabolic and molecular methods.

Materials and methods

Bacterial strains and culture medium

The strain C613 used in this study had been previously isolated from a sample of paper affected by foxing (Lippi et al., 1995). *Bacillus circulans* (NCIMB 9555), *Bacillus coagulans* (DSMZ 1), *Bacillus laevolacticus* (DSMZ 442), *Paenibacillus macerans* (DSMZ 24) and *Paenibacillus polymyxa* (DSMZ 36) were used as reference strains in the different analyses. All the bacteria were routinely cultured and maintained on nutrient Broth medium (NB) containing per liter: 5 g peptone and 3 g beef extract. When necessary, the medium was solidified with 1.8% agar (NA).

Biolog carbon substrates utilization patterns

Biolog GP2 MicroPlates (Biolog, Inc., Hayward, CA, USA) were inoculated in duplicate using the standard procedures (Garland and Mills, 1991) with either C613 strain or *B. circulans* and were incubated at 30 °C. The optical density at 590 nm