NOTE

Reclassification of bioindicator strains *Bacillus subtilis* DSM 675 and *Bacillus subtilis* DSM 2277 as *Bacillus atrophaeus*

Dagmar Fritze and Rüdiger Pukall

Author for correspondence: Dagmar Fritze. Tel: +49 531 2616 254. Fax: +49 531 2616 418.
e-mail: dfr@dsmz.de

DSMZ–Deutsche Sammlung
von Mikroorganismen und
Zellkulturen GmbH,
Mascheroder Weg 1b,
D-38124 Braunschweig,
Germany

On the basis of high DNA–DNA reassociation values and confirmatory automated RiboPrint analysis, two aerobic spore-forming strains hitherto allocated to *Bacillus subtilis* and used as bioindicators (DSM 675, hot-air sterilization control; DSM 2277, ethylene oxide sterilization control) are reclassified as *Bacillus atrophaeus*.

Keywords: sterilization control, '*Bacillus globigii*', red strain, '*Bacillus subtilis* var. *niger*', *Bacillus atrophaeus*

Strains of the species *Bacillus subtilis* are used in a variety of applications, an important one being sterilization control. Strains of this species produce spores of specific resistance to, for example, dry heat or ethylene oxide and are thus proposed for testing the effectiveness of such methods for sterilization (Kelsey, 1967; Russell *et al.*, 1992; US Pharmacopeia, 1995; CEN–European Committee for Standardization, 1997a, b). *B. subtilis* DSM 675, originally designated as the 'red strain', was especially suited for routine use because of its distinctly coloured colonies.

Modern taxonomic methods have led to numerous reclassifications and rearrangements of strains, species and genera. This has been particularly true for the genus *Bacillus*, which has undergone a wide range of taxonomic developments in recent years. Most of these investigations are usually based on type strains; only rarely are additional strains of the species in question included. Thus, strains of practical importance, e.g. test and control strains, are often not taken into account.

The long history of strain DSM 675, the 'red strain'

In 1900, Migula described the species '*Bacillus globigii*'. When Smith *et al.* (1952) re-examined a number of strains received under this name, they had to allocate all of them to other more established species. Strains with traits corresponding to the original description were transferred to *Bacillus licheniformis*, because the original description of '*B. globigii*' by Migula was judged to be synonymous with that for *B. licheniformis*. Those strains not corresponding to the original description were allocated to *Bacillus*

circulans, *Bacillus pumilus* and '*B. subtilis* var. *niger*'. Two strains from the Bacon Laboratories (the 'red strain' and the 'brown strain') were allocated to the latter species and were designated as NRS-1221A and NRS-1221B, respectively. In the same work, the authors concurrently reduced '*Bacillus niger*' from species to variety because they had found no discriminatory property, other than pigmentation, between *B. subtilis*, '*Bacillus atterimus*' and '*B. niger*'. This property was shown to be susceptible to culture conditions (e.g. cultivation on media containing glucose or cultivation at a high incubation temperature). Clarifying the situation, Smith *et al.* (1952) stated (p. 83) that "the characterization of *B. subtilis* serves for '*B. subtilis* var. *niger*' by adding the words substrate blackened to the description of the growth on mediums containing tyrosine".

Later, Gordon *et al.* (1973) found 'varieties' unsatisfactory and subsumed them under *B. subtilis* knowing that this was a 'lumped' group; this group, with the arrival of better tests and methods, could then be taken apart again and 'good' species described. Indeed, since then, a number of new species have been separated from the species *B. subtilis sensu stricto* and validly published (Priest *et al.*, 1987; Nakamura, 1989; Roberts *et al.*, 1994, 1996; Nakamura *et al.*, 1999).

Nakamura (1989) re-examined the black-pigment-producing strains of *B. subtilis* and, on the basis of pigment production (on two different media) and DNA hybridization studies, he was able to discriminate between three groups of strains. Group 3 did not produce any pigment on either medium and included the type strain of *B. subtilis*. Group 2 was a pigment-forming variant but still belonged to *B. subtilis sensu*

Table 1. *Bacillus* strains investigated in this study

ATCC, American Type Culture Collection; BMTU, Boehringer Mannheim Tutzing; CCM, Czech Collection of Microorganisms; CIP, Collection de l'Institut Pasteur; DSM, DSMZ–Deutsche Sammlung von Mikroorganismen und Zellkulturen; IFO, Institute for Fermentation, Osaka; NCDO, National Collection of Dairy Organisms; NCIB, National Collection of Industrial Bacteria; NCTC, National Collection of Type Cultures; NRRL, Northern Regional Research Laboratory; NRS, Nathan R. Smith.

DSM no.	History	Other collection nos
<i>B. subtilis</i> DSM 675	← BMTU ← ATCC ← N. R. Smith (1221A, ' <i>B. subtilis</i> var. <i>niger</i> ') ← Frederick S. Bacon Laboratories, Watertown, Massachusetts, 1947 (' <i>Bacillus globigii</i> ', 'red strain') ← C. R. Phillips, Fort Detrick, USA ← Elisabeth McCoy	ATCC 9372, NCIB 8058, CIP 77.18 NRS 1221A, IFO 13721, NCDO 738
<i>B. subtilis</i> DSM 2277	← NCTC ← J. C. Kelsey, London ← C. R. Phillips, Fort Detrick, USA (' <i>B. globigii</i> ') ← NRRL ← NRS-213 (' <i>B. subtilis</i> var. <i>niger</i> ') ← ATCC ← H. J. Conn, strain Marburg	NCTC 10073, NCIB 8649, CIP 103406 NRRL–NRS 213 ^T , ATCC 49337 ^T NRS 744 ^T , ATCC 6051 ^T , CCM 2216 ^T , NCIB 3610 ^T , NCTC 3610 ^T , IFO 12210 ^T
<i>B. atrophaeus</i> DSM 7264 ^T		
<i>B. subtilis</i> DSM 10 ^T		

Table 2. Percentage DNA–DNA similarity

The DNA–DNA similarity values are the means of at least two determinations.

Strain	DSM 2277	DSM 675	DSM 7264 ^T	DSM 10 ^T
DSM 2277	—	87	98	30
DSM 675		—	88	32
<i>B. atrophaeus</i> DSM 7264 ^T			—	ND
<i>B. subtilis</i> DSM 10 ^T				—

ND, Not determined.

stricto according to the high DNA–DNA similarity values between groups 2 and 3. Both groups (2 and 3) represent the species *B. subtilis*. Group 1, which produced a brownish-black pigment on one medium and a brown pigment on the other, showed low levels of DNA hybridization with groups 2 and 3. Thus, group 1 was described as the new species *Bacillus atrophaeus*. Twenty-one of the 25 strains in this group had previously been designated as '*B. subtilis* var. *niger*'.

Unfortunately, neither '*B. subtilis* var. *niger*' DSM 675 (or any of its equivalents in other collections) nor '*B. subtilis* var. *niger*' DSM 2277 was included in this study. To reveal the taxonomic position of these important sterilization control strains, spectroscopic DNA–DNA hybridizations (Huß *et al.*, 1983) and automated RiboPrint (Qualicon) analyses (Bruce, 1996) were performed on all relevant strains (Table 1, Table 2, Fig. 1).

The present study reveals high DNA–DNA homology values between the two strains and the type strain of *B. atrophaeus* (DSM 7264^T) and low hybridization values with *B. subtilis* DSM 10^T. In addition, RiboPrint patterns for all of the strains involved were generated

Fig. 1. Normalized RiboPrint pattern found within '*Bacillus subtilis*' strains DSM 675 and DSM 2277, related to the type strain of *Bacillus atrophaeus*, compared with the ribotype pattern of the type strain of *B. subtilis*.

and compared with each other and with other *Bacillus* type strains. Strains DSM 675 and DSM 2277 showed a close association with *B. atrophaeus*, and a separation from *B. subtilis* was confirmed (the similarity coefficients of the RiboPrint patterns were approximately 0.92 and 0.94, respectively; see Fig. 1).

Thus, both sterilization control strains DSM 675 and DSM 2277, previously named '*B. globigii*', '*B. niger*', '*B. subtilis* var. *niger*' and, finally, *B. subtilis*, have to be reclassified as members of the species *B. atrophaeus*. Species descriptions of *B. subtilis* and *B. atrophaeus* are not affected by this reclassification, as Smith *et al.* (1952) had classified the 'red strain' as '*B. subtilis* var. *niger*' after its substrate blackening of media containing tyrosine. Nakamura (1989) described the soluble pigment as 'brownish black' or 'dark brown' and stated that 'except for the colour of the soluble pigment, all of the strains were indistinguishable by the standard characterization method; i.e. they exhibited the traits typical of *B. subtilis*'.

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