Phylogenetic analysis of transformable strains of thermophilic Bacillus species

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Abstract

Few strains of thermophilic Bacillus spp are readily transformable with plasmid DNA. Given the considerable phylogenetic and phenotypic diversity amongst thermophilic bacilli, we have examined whether transformability is a trait associated with a particular phylogenetic group, by sequencing the 16S ribosomal RNA genes from transformable strains NUB3621, K1041, and NRRL1174. Although all of these strains were described in the literature as B. stearothermophilus, only NRRL1174 is closely related to the type strain of this species. Based on its 16S rDNA sequence and physiological data K1041 appeared to belong to the species B. thermodenitrificans, while NUB3621 showed a slightly closer relationship to B. thermoglucosidasius than to B. stearothermophilus. Therefore we conclude that the trait of transformability, though possibly strain-specific, is not limited to a single species of thermophilic Bacillus.

Keywords: Bacillus stearothermophilus; Thermophile; Phylogeny; 16S rDNA

1. Introduction

Thermophilic Bacillus species, with growth temperature optima between 45°C and 70°C, have been isolated from a wide range of environments and are important contaminants of heat-treated food products [1]. Research interest in these organisms has increased due to their biotechnological potential, especially as sources of thermostable enzymes [2] and as potential ethanologens [3]. Progress has been assisted by the discovery that protoplasts of some thermophilic strains can be transformed with plasmid DNA [4–7] which has allowed the over-expression of thermostable enzymes such as amylases [8–10], neutral protease [11], penicillinase [12] and carboxymethylcellulase [13] in thermophilic hosts. Furthermore Liao et al. [7] successfully isolated a thermostable kanamycin nucleotidyl transferase by ‘forced evolution’ using B. stearothermophilus NRRL1174 as a host.

According to the literature, four strains of B. stearothermophilus have been transformed with plasmid DNA using adaptations of Chang and Cohen’s [14] protoplast/polyethylene glycol method. These strains are CU21, SIC1, NRRL1174 and NUB36 (and several mutants derived from this strain). Welker and colleagues have made considerable progress in developing genetic techniques for B. stearothermophilus NUB36. Not only have they trans-
formed NUB36 protoplasts with plasmid DNA [6] and intact cells with phage DNA [15] but have also isolated a series of auxotrophic mutants [16] and constructed a linkage map of the chromosome as a result of protoplast fusion experiments [17]. Narumi et al. [18] transformed intact cells of *B. stearothermophilus* K1041 with plasmid DNA by electroporation. This procedure is more convenient and reproducible as, once prepared, electrocompetent cells could be stored frozen for later use, while protoplasts need to be freshly prepared and their formation and handling can be problematic due to their fragility. Despite the success with these strains it is evident that many strains of thermophilic *Bacillus* spp cannot be readily transformed. While screening for suitable transformable strains for genetic manipulation it became evident that at least some of the strains cited above had been mis-classified. This raised the question as to whether transformability may be species-specific or a trait associated with a particular phylogenetic group, which is the subject of this paper.

Recently phylogenetic relationships among the genus *Bacillus*, including thermophilic species, have been explored by analysis of 16S rDNA sequences. Ash et al. showed that [19] the *Bacillus* species formed five main phylogenetic groups. One of these groups, group 5, comprised the thermophilic species *B. stearothermophilus*, *B. thermoglucosidasius*, and *B. kaustophilus*. Two moderately thermophilic species *B. smithii* and *B. coagulans* fell into group 1 along with *B. subtilis* and other mesophilic species.

As the transformable strain CU21 was derived from ATCC12890, the type strain of *B. stearothermophilus* [4] its classification was not in question, while strain SIC1 was not available for this study. Here we present evidence that strains NUB3621 (and hence its parent, NUB36) and K1041 do not belong to the species *B. stearothermophilus* but are more closely related to *B. thermoglucosidasius* and *B. thermodenitrificans* respectively.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Thermophilic strains K1041, NUB3621, and NRR1174 were kindly given by I. Narumi (Japan Atomic Energy Research Institute, Takasaki, Japan), N. Welker (Northwestern University, Evanston, USA), and H. Liao (Cangene, Ontario, Canada) respectively. NUB3621 is a rifampicin-resistant derivative of NUB36, and lacks the parent strain’s restriction and modification system. *B. stearothermophilus* NCA1503 was purchased from NCIMB (National Collection of Industrial and Marine Bacteria, Aberdeen, UK). Stock cultures were streaked onto L-agar plates (10 g/l Bacto Tryptone, 5 g/l Bacto Yeast Extract, 10 g/l NaCl, 15 g/l Bacto Agar, pH 7.3) and were incubated at 52°C.

2.2. Oligonucleotide primers

Synthetic oligonucleotides were purchased from MWG Biotech (Milton Keynes, UK).

Primers 27F (GAGAGTTTGATCCTGGCTCA-G), 765R (CTGTTTGCTCCCCACGCTTC), and 1495R (CTACGGCTACCTGGTTACGA) were universal primers for eubacterial 16S rDNA [20]. Three additional primers were designed on the basis of alignments of *Bacillus* 16S rDNA sequences: 400F (GGAGCGACGCCGCGTGAGCG), 700F (GCAACTGACGCTGAGGCG), and 1000F (GCAACGCGAAGAACCTTA).

2.3. Polymerase chain reaction (PCR)

16S rDNA was amplified by PCR directly from freshly grown overnight colonies using Bio-X-act thermostable DNA polymerase (Bioline, London, UK). PCR products were purified from agarose gels using the Wizard PCR Preps purification system (Promega, Southampton, UK).

2.4. DNA sequencing

Dye Terminator Sequencing was carried out using AmpliTaq DNA polymerase FS with an ABI373 automated sequencer (Perkin Elmer Applied Biosystems, Foster City, USA).

2.5. Physiological tests

The ability to produce acid from various carbohydrate substrates was tested using CH50 API strips
(BioMérieux, Marcy l’étoile, France). Production of gas from nitrate was tested using API 20E strips (BioMérieux). The API strip tests were carried out as directed by the manufacturer except that all incubations were at 52°C. These tests were carried out in triplicate using *B. stearothermophilus* NCA1503 [21] as a control strain.

### 2.6. Nucleotide sequence accession numbers

The 16S rDNA sequences amplified from NUB3621 and K1041 have been deposited in the EMBL Data Library (Accession numbers AJ005759 and AJ005760 respectively).

### 3. Results and discussion

The 16S rRNA genes were amplified from strains K1041, NUB3621, and NRRL1174 by PCR using primers 27F and 1495R and initially sequenced using primer 27F. The 16S rDNA sequence obtained for NRRL1174 was almost identical to the published sequence for *B. stearothermophilus* ATCC12890 [22] but the sequences from K1041 and NUB3621 contained notable differences from that of ATCC12890. Therefore we further sequenced the 16S rDNA from K1041 and NUB3621 using primers 27F, 400F, 700F, 765R, 1000F, and 1495R. The 16S rDNA sequences amplified from NUB3621 and K1041 were aligned with other published sequences from group 5 *Bacillus* strains [19,22,23] using CLUSTALW [24]. Since there was some variation in length of sequences deposited in the databases, the alignment was trimmed at each end to give an alignment 1411 nucleotides in length, from which percentage identities were calculated. Since these sequences were all closely related (87% to 99% identical), there were few insertions and deletions and there was very little ambiguity in the alignment. Manual editing of the few ambiguously aligned residues resulted in no change in the pattern of relationships between the sequences from K1041 and NUB3621 with the other sequences. The sequence from K1041 most closely resembled those from *B. thermodenitrificans* (98 and 99% sequence identity to strains DSM465 and DSM466 respectively) and shared only 93% identity with that from *B. stearothermophilus* ATCC12890.

These same percentage identities were obtained when the 41 gaps were excluded from the alignment. The most similar sequences to that from NUB3621 were those from *Bacillus* sp ICPS6 (97% identity) and *B. thermoglucosidasius* KP1006 (95% identity), with only 90% identity to the *B. stearothermophilus* ATCC12890 sequence. When the gaps were excluded, the NUB3621 sequence was 92, 92, and 97% identical to those from *B. stearothermophilus* ATCC12890, *B. thermoglucosidasius*, and strain ICPS6 respectively. These relationships were further illustrated by cre-
ating a phylogenetic tree. One hundred bootstrapped data sets were generated and pairwise distances calculated according to the Jukes and Cantor [25] model using programs SEQBOOT and DNADIST from the PHYLIP package [26,27]. This distance matrix was then used to construct the consensus tree in Fig. 1 by the neighbour-joining method with NEIGHBOR and CONSENSE from the PHYLIP package. The tree was rooted with *B. smithii* as the outgroup and illustrated using TREEVIEW [28].

The DNAPARS program from the PHYLIP package was also used to find the most parsimonious trees from the aligned sequences. Ten equally parsimonious trees were found. The positions of K1041 and NUB3621 were consistent in all the ten trees and equivalent to their positions in the tree illustrated in Fig. 1.

According to Welker and colleagues [15], NUB36 was identified as *B. steaothermophilus* on the basis of the criteria set out by Gibson and Gordon [29] and Smith et al. [30]. However in the light of more recent studies [19,22,31,32] many strains previously described as *B. steaothermophilus* on this basis have been assigned to other *Bacillus* species. Certainly the classification of K1041 to the species *B. steaothermophilus* on this basis has been assigned to other *Bacillus* species. The classification of K1041 to the species *B. steaothermophilus* [15] appears erroneous. Narumi et al. assigned it to Walker’s and Wolf’s [32] group 1b.

White et al. [31] re-examined many of Walker’s and Wolf’s strains more thoroughly and re-classified them taking into account the developments in the taxonomy of thermophilic bacilli over the previous two decades. Of those strains examined which belonged to Walker’s and Wolf’s group 1b1, White et al. [31] assigned none to *B. steaothermophilus sensu stricto*. Therefore the classification of K1041 as a member of the species *B. steaothermophilus* seems dubious.

As discussed above, 16S rDNA analysis indicates a close phylogenetic relationship between K1041 and *B. thermodenitrificans*. However, White et al. [31] revealed several, as yet unnamed, taxa within the thermophilic bacilli, which appeared to be related to, but distinct from the named species. As 16S rDNA sequences are not available for these taxa, it remains possible that K1041 may have a closer phylogenetic relationship to one of these. White et al. [31] listed the following characteristics of *B. thermodenitrificans*: ability to hydrolyse starch, RNA, tributyrin and xylan; nitrate and nitrite reduced to acid; acid produced from arabinose, cellobiose, melezitose, melibiose and trehalose but not from galactose or rhamnose; flat colonies with a fimbriate margin. Colonies of K1041 on L-agar were flat with a fimbriate margin. These colonies were rather dry in appearance, a trait shared with *B. thermodenitrificans* strains DSM465 and DSM466 whereas most thermophilic *Bacillus* strains form ‘shiny’ wet colonies (unpublished observations). Consistent with its classification as *B. thermodenitrificans*, we found that strain K1041 produced acid from starch, arabinose, cellobiose, melezitose, melibiose, and trehalose but only very weakly produced acid from galactose and rhamnose. Furthermore K1041 reduced nitrate to gas. Therefore, on the basis of both 16S rDNA and biochemical data, we believe that K1041 should be assigned to *B. thermodenitrificans*.

The proper classification of NUB3621 is of some scientific significance since its parent strain, NUB36, has been the subject of considerable genetic analysis including mapping of the chromosome [17]. NUB36 was assigned to the species *B. steaothermophilus* [17] primarily on the basis of its growth at 65°C which, in the light of recent studies and re-examination of the phylogeny and taxonomy of the thermophilic bacilli, is clearly inadequate. The 16S rDNA sequence analysis demonstrated that NUB3621 (and therefore its parent NUB36) was phylogenetically slightly more closely related to *B. thermoglucosidasius* than to *B. steaothermophilus* or any other named species. Therefore it might be preferable to assign this strain to *B. thermoglucosidasius* rather than to *B. steaothermophilus*. However in the work of White et al. [31] there were several taxa which were related to, but distinct from *B. thermoglucosidasius*. Furthermore a thermophile having a very similar 16S rDNA sequence to that of *B. thermoglucosidasius* was recently proposed as a separate species, *B. thermoantarticus* [33], on the basis of its unusually high G+C content. Therefore until the limits of the variation circumscribed by each species are better defined, the classification of NUB3621 (and NUB36) as *B. thermoglucosidasius* can only be tentative.

On the basis of these results, it is evident that transformability is not a species-specific trait, being found in *B. steaothermophilus* (CU21 and NRRL1174), *B. thermoglucosidasius* (NUB3621)
and *B. thermodenitrificans* (K1041). However, it may be that certain species are not transformable so it would be worth extending this study to establish whether this trait associates with certain groups. Furthermore, as the transformable strains are likely to predominate in future genetic studies an accurate phylogenetic association should be valuable.

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**References**


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