NOTE**Phylogeny of marine and freshwater *Shewanella*: reclassification of *Shewanella putrefaciens* NCIMB 400 as *Shewanella frigidimarina***

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Dissimilatory Fe(III) reduction by *Shewanella putrefaciens* and related species has generated considerable interest in biochemical characterization of the pathways for anaerobic electron transfer in this organism. Two strains, MR-1 and NCIMB 400, have been extensively used, and several respiratory enzymes have been isolated from each. It has become apparent that significant sequence differences exist between homologous proteins from these strains. The 16S rRNA from NCIMB 400 was sequenced and compared to the sequences from MR-1 and other *Shewanella* strains. The results indicate that NCIMB 400 is significantly more closely related to the newly identified *Shewanella frigidimarina* than to the *S. putrefaciens* type strain. It is therefore proposed that NCIMB 400 should be reclassified as *S. frigidimarina*.

Keywords: *Shewanella*, 16S rRNA, cytochromes, anaerobic respiration

Shewanella putrefaciens and its close relatives are Gram-negative, facultative anaerobes that exhibit an enormous diversity of metabolism that is particularly apparent during anaerobic respiration. In the absence of oxygen, *S. putrefaciens* can couple growth to the reduction of at least ten different terminal electron acceptors, including nitrate, nitrite, fumarate, thio-sulphate, elemental sulphur (S⁰), trimethylamine *N*-oxide and, more unusually, the particulate metal oxides Mn(IV) and Fe(III) (Myers & Nealson, 1988; Moser & Nealson, 1996). The ability to use insoluble metal oxides has generated substantial interest in this group of organisms but the pathway for Mn(IV) and Fe(III) reduction is poorly understood, particularly since it seems that redox enzymes must be located in the outer membrane, exposed to the extracellular medium, so that they can contact the particulate electron acceptor. Anaerobic growth results in the synthesis of several novel cytochromes (Morris *et al.*, 1990), some of which have been purified and characterized biochemically and by sequence determination. It has been reported that some cytochromes are

localized to the outer membrane (Myers & Myers, 1992).

Shewanella sp. MR-1 was isolated from the freshwater sediment of Oneida Lake, New York, USA (Myers & Nealson, 1988), whereas NCIMB 400 is a marine organism that was isolated from the North Sea near Aberdeen, UK (Lee *et al.*, 1977). Both of these organisms produce a soluble, periplasmic flavocytochrome *c*₃ fumarate reductase that is required for fumarate respiration (Gordon *et al.*, 1998; Myers & Myers, 1997) and that is very different from other bacterial fumarate reductases (Pealing *et al.*, 1992).

The 16S rRNA gene sequence was determined for *Shewanella* strain NCIMB 400 by PCR amplification of genomic DNA using primers pA and pH* as described by Edwards *et al.* (1989). The 1.5 kb product was cloned and the sequence was determined (Sequenase 2.0 kit dITP). The accession number for this sequence is Y13699. The 16S rDNA sequences of 22 other *Shewanella* strains were manually aligned with this sequence. The PHYLIP suite of programs (Felsenstein, 1993) was then used to analyse the data. Bootstrap analysis (1000 replicates) was used to test the tree obtained. The accession numbers of the sequences used in this study are as follows: *Shewanella frigidimarina* ACAM 593, U85904; *S. frigidimarina* ACAM 600, U85906; *S. frigidimarina* ACAM 588,

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The EMBL accession number for the 16S rRNA gene sequence of strain NCIMB 400 reported in this paper is Y13699.

Fig. 1. Unrooted phylogenetic tree based on 16S rRNA comparisons showing the relationship of NCIMB 400 to other *Shewanella* strains. The branching pattern was generated by neighbour-joining methods and the bootstrap values, shown at the nodes, were calculated from 1000 replicates.

U85905; *S. frigidimarina* ACAM 591^T, U85903; *S. frigidimarina* ACAM 584, U85902; *Shewanella* sp. SC2A, U91591; *S. frigidimarina* ACAM 122, U39398; *Shewanella algae* BrY, X81621; *S. putrefaciens* NCIMB 10471^T, X82123; *Shewanella* sp. MR-30, AF005255; *Shewanella* sp. MR-1, AF005251; *Shewanella* sp. MR-8, AF005254; *Shewanella* sp. MR-4, AF005252; *Shewanella* sp. MR-7, AF005253; *Shewanella amazonensis* SB2B, Z95845; *S. algae* FeRed, X81622; *S. algae* ATCC 51192^T, Z95846; *Shewanella* sp. DB172r, D63488; *Shewanella* sp. DB172f, D63824; *Shewanella benthica* ATCC 43992, X82131; *Shewanella gelidimarina* ACAM 456^T, U85907; and *Shewanella hanedai* ATCC 35256, U91589.

A PCR approach (Edwards *et al.*, 1989) was used to isolate 1530 bp from the 16S rRNA gene of NCIMB 400 and its complete sequence was determined. This sequence was compared with the corresponding sequences of several other *Shewanella* species. These sequences are all quite closely clustered and clearly

Fig. 2. Alignment of the N-terminal amino acid sequences of cytochrome c_3 from NCIMB 400 and MR-1. The N-terminal sequence of cytochrome c_3 from NCIMB 400 was determined by direct amino acid sequence and confirmed by sequencing the DNA encoding this protein (E. H. J. Gordon, A. D. Pike, H. Fischer, S. K. Chapman & G. A. Reid, unpublished results; EMBL no. AJ000006). The sequence of MR-1 cytochrome c_3 is taken from Tsapin *et al.* (1997).

distinct from those of other bacterial genera. However, it appears that the NCIMB 400 sequence is substantially more similar to the 16S rRNA sequences from several newly described strains from Antarctic sea ice that have been assigned to a new species, *S. frigidimarina* (Bowman *et al.*, 1997), than it is to others, including the *S. putrefaciens* type strain and MR-1 (Fig. 1). Pairwise comparisons showed 98.5–99.5% sequence identity over 1.5 kb between the NCIMB 400 sequence and those from the *S. frigidimarina* strains. In contrast, the sequences from NCIMB 400 and MR-1 are 95.7% identical.

Bowman *et al.* (1997) found that *S. frigidimarina* DNA has a G + C content of 40–43 mol% whereas *S. putrefaciens* DNA has a value of 44–47 mol%. The value for NCIMB 400 (Lee *et al.*, 1977) is 41.7 mol%, which clearly falls into the range for *S. frigidimarina*.

Several cytochromes have been identified and isolated from MR-1 and NCIMB 400. In general, these proteins share similar biochemical and biophysical properties but few have been subjected to detailed molecular analysis. A small, periplasmic cytochrome c_3 has been purified from each of these organisms after anaerobic growth and subjected to N-terminal sequencing (Tsapin *et al.*, 1997; Gordon *et al.*, 1998). The aligned sequences (Fig. 2) match at only 12 of 19 positions (63%) indicating a remarkable divergence in at least this respiratory protein and emphasizing the need to distinguish between the two strains in the literature. Both strains have generally been referred to as *S. putrefaciens*, but it is proposed that NCIMB 400 should be reclassified as *S. frigidimarina*.

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