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### Publication Date

2007-02-01

Peer reviewed



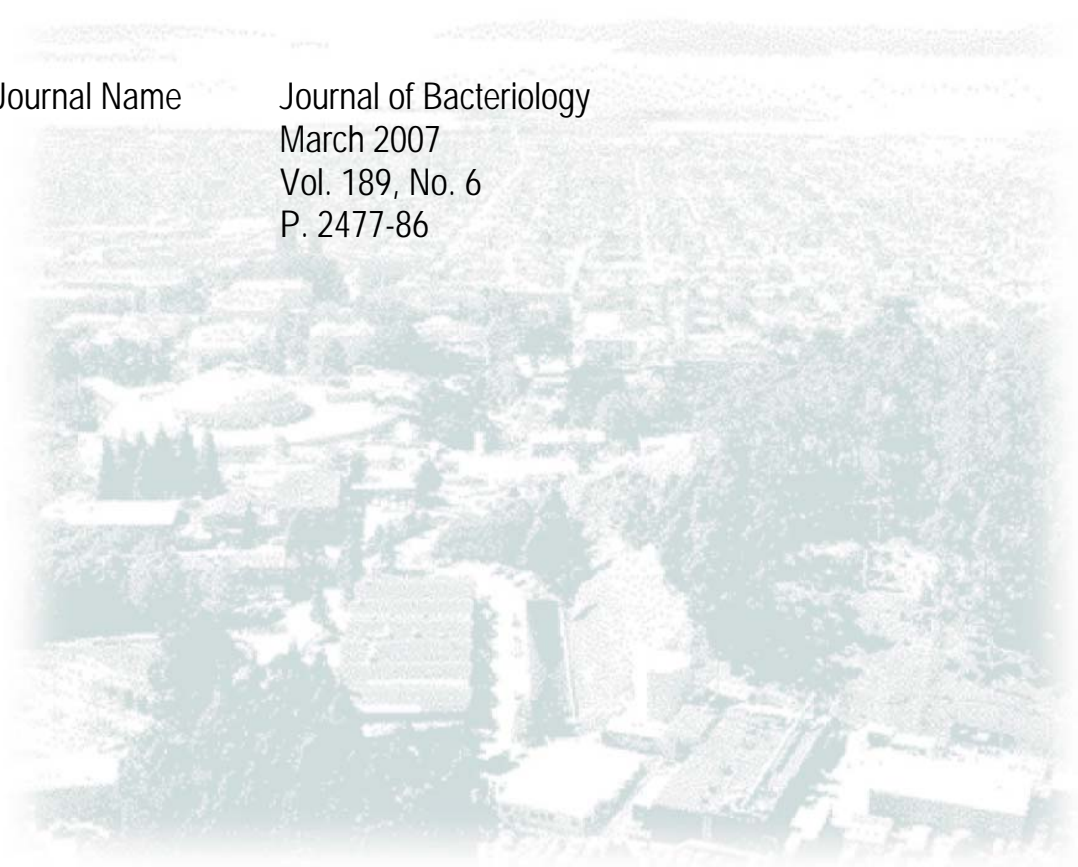
## ERNEST ORLANDO LAWRENCE BERKELEY NATIONAL LABORATORY

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Division Genomics

Journal Name Journal of Bacteriology  
March 2007  
Vol. 189, No. 6  
P. 2477-86



1 **Genome Sequence and Analysis of the Soil Cellulolytic Actinomycete *Thermobifida fusca***

2 **YX.**

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17 Running title: Genome sequence of cellulolytic *T. fusca*  
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19 Keywords: *Thermobifida fusca*, cellulase, xylanase, genome

1 **ABSTRACT**

2 *Thermobifida fusca* is a moderately thermophilic soil bacterium that belongs to *Actinobacteria*.  
3 It is a major degrader of plant cell walls and has been used as a model organism for the study of  
4 secreted, thermostable cellulases. The complete genome sequence showed that *T. fusca* has a  
5 single circular chromosome of 3642249 bp predicted to encode 3117 proteins and 65 RNA  
6 species with a coding density of 85%. Genome analysis revealed the existence of 29 putative  
7 glycoside hydrolases in addition to the previously identified cellulases and xylanases. The  
8 glycosyl hydrolases include enzymes predicted to exhibit mainly dextran/starch and xylan  
9 degrading functions. *T. fusca* possesses two protein secretion systems: the *sec* general secretion  
10 system and the twin-arginine translocation system. Several of the secreted cellulases have  
11 sequence signatures indicating their secretion may be mediated by the twin-arginine  
12 translocation system. *T. fusca* has extensive transport systems for import of carbohydrates  
13 coupled to transcriptional regulators controlling the expression of the transporters and  
14 glycosylhydrolases. In addition to providing an overview of the physiology of a soil  
15 actinomycete, this study presents insights on the transcriptional regulation and secretion of  
16 cellulases which may facilitate the industrial exploitation of these systems.

## 1 INTRODUCTION.

2 *Thermobifida fusca* (formerly known as *Thermomonospora fusca*) is an aerobic, moderately  
3 thermophilic, filamentous soil bacterium that is a major degrader of plant cell walls in heated  
4 organic materials such as compost heaps, rotting hay, manure piles or mushroom growth medium  
5 (1). It produces spores that can be allergenic and has been associated with a condition called  
6 farmers lung (50). Its extracellular glycoside hydrolases (cellulases and xylanases) have been  
7 studied extensively because of their thermostability, broad pH range (4-10) and high activity. It  
8 appears to degrade all major plant cell wall polymers except lignin and pectin and can grow on  
9 most simple sugars and carboxylic acids. It belongs to the phylum of *Actinobacteria* and was  
10 first isolated from decaying wood (2).

11 *T. fusca* has been the source organism for isolating and studying multiple secreted cellulases and  
12 other carbohydrate degrading enzymes ((12, 15). Using classical biochemical methods six  
13 different cellulases have been identified: four endocellulase genes (7, 12, 15) and two  
14 exocellulases (18, 53). In addition, an intracellular  $\beta$ -glucosidase that degrades cellobiose to  
15 glucose(46), an extracellular xyloglucanase (17) two secreted xylanases and a GH family 81  
16  $\beta$ -1,3-glucanase (4, 11, 16, 22, 31) have been cloned and characterized. Secreted cellulases  
17 have great biotechnological promise for utilization in the degradation of agricultural products  
18 and waste to produce sugars that can be subsequently converted to ethanol. Several complete  
19 genomic sequences of the phylum *Actinobacteria* are currently available. The availability of  
20 these complete genomic sequences of the phylum *Actinobacteria* (29) enables sequence  
21 comparisons, which can provide valuable information for the biotechnological application of  
22 these microbes.

23

## 24 MATERIALS AND METHODS.

1 **Genome Sequencing and Assembly.** The complete genome of *T. fusca* was sequenced at the  
2 Joint Genome Institute using a combination of 3kb and fosmid (40kb) libraries. Library  
3 construction, sequencing, finishing and automated annotation steps were performed as described  
4 at the JGI webpage (<http://www.jgi.doe.gov/sequencing/index.html>). Predicted coding  
5 sequences (CDSs) were manually analyzed and evaluated using an Integrated Microbial  
6 Genomes (IMG) annotation pipeline (<http://img.jgi.doe.gov>).

7 **Genome analysis.** Comparative analysis of *T. fusca* with related organisms was performed  
8 using a set of tools available in IMG. Unique and orthologous *T. fusca* genes were identified by  
9 using BLASTp (cutoff scores of  $E < 10^{-2}$  and 20% identity and reciprocal hits with cutoff scores  
10 of  $E < 10^{-5}$  and 30% identity, respectively). Signal peptides were identified using the SignalP 3.0  
11 (3) and TMHMM (25) at default values. Whole genome comparisons were performed using  
12 MUMmer (27).

13 **GenBank accession numbers.** The sequence data described here have been deposited in  
14 GenBank (CP000088).

## 15 **RESULTS AND DISCUSSION.**

16 **Genome features and comparative actinobacteria genomics.** The *T. fusca* genome consists of  
17 a single circular chromosome with 3642249 bp. The GC content is 67.5% and there are 3117  
18 predicted CDSs in the genome. The overall genome statistics are listed in Figure 1. Among the  
19 predicted genes 68% have been assigned a function. 26% (830 genes) display sequence  
20 similarity to other organisms in the database with no known function and 106 genes (3.3%)  
21 appear to be unique in *T. fusca*. There are four *rrn* loci arranged in 5S-23S-16S operons.  
22 According to Suhre and Claverie (47), thermophilic proteomes exhibit an increased content of  
23 charged (Asp, Glu, Lys, Arg) versus polar (Asn, Gln, Ser, Thr) residues. This trend is a common  
24 feature for organisms with optimal growth temperature higher than 55 °C. *T. fusca* exhibits the  
25 same trend with the striking exception of Lys residues, which appear to be close to the minimum

1 for bacteria in IMG. The reverse trend is observed for Ala which is elevated in *T.fusca* while  
2 most thermophiles have fewer Ala residues (Figure 2).

3 *T. fusca* has 412 (~13%) unique genes when compared to the 32 Actinobacteria genomes present  
4 in IMG. From these 412 genes only 83 CDSs have InterPro hits and the rest are hypothetical  
5 proteins with no functional hits. Comparisons between representatives of the five major  
6 *Actinobacteria* genera, *T. fusca*, *S. coelicolor*, *M. tuberculosis*, *Nocardia farcinica* and  
7 *Corynebacterium diphtheriae* indicate that there are 1101 genes (~30%) of genes shared between  
8 these five organisms. *T. fusca* has 660 unique genes (20%) when compared to the above five  
9 genomes. Overall, comparisons between these five genomes both in terms of gene similarity  
10 (Table 1) and synteny (Figure 3) indicate that *T. fusca* is closest related to *S. coelicolor* and *N.*  
11 *farcinica*.

12 **Cellulases and glycoside hydrolases.** The genome of *T. fusca* encodes a total of 45 hydrolytic  
13 enzymes predicted to act on oligo- and/or poly-saccharides as identified by the CAZy ModO  
14 database, <http://afmb.cnrs-mrs.fr/CAZY/> (Table 2). These enzymes include 36 glycoside  
15 hydrolases, 9 carbohydrate esterases and 2 polysaccharide lyases. The glycoside hydrolases are  
16 distributed in 22 families (GH families), with the GH\_13 family being the most abundant with  
17 six individual members represented in the genome. Computational analysis of signal peptides  
18 predicts 16 glycoside hydrolases are secreted. The majority of these secreted proteins (13 out of  
19 16) have a signal sequence resembling the twin-arginine translocation (TAT) signal in their N-  
20 terminus. Analysis of the secretion systems will be presented in a subsequent section.

21 Fourteen enzymes have been isolated and studied (including six enzymes with various  
22 cellulolytic activities, cellulases E1 to E6, as well as a beta mannanase and an endo xylanase)  
23 from *T. fusca* (Table 2). In addition to these enzymes 28 more putative glycosyl hydrolases and  
24 enzymes potentially involved in plant cell wall degradation were identified in the genome  
25 (mainly with dextran/starch and xylan degrading functions). Two enzymes were identified to be

1 similar to endochitinase and exochitinase (Tfu0580 and Tfu0868 respectively). The absence of  
2 N-acetyl-hexosaminidase implies that chitin degradation proceeds either with an alternative  
3 pathway or by gradual degradation of the substrate by an exochitinolytic enzyme. Deacetylases  
4 of chitin, xylan and N-acetyl-glucosamine were identified suggesting that the organism can  
5 utilize these substrates for energy production. *T. fusca* contains several enzymes involved in the  
6 degradation of xylan: endo- $\beta$ -1,4-xylanase (Tfu1213,Tfu2791,Tfu2923); xylosidase, (Tfu1613);  
7  $\alpha$ -L-arabinofuranosidase (Tfu1616); and several CDSs with putative acetylxylan esterase  
8 activity. In addition to the above mentioned carbohydrases *T. fusca* possesses two CDSs with  
9 significant similarity to pectate lyases, Tfu0153 and Tfu2168 (Table 2). These two genes encode  
10 secreted proteins that possess a signal peptide that may be interacting with the TAT system.  
11 Pectate lyases (EC 4.2.2.2) catalyze the eliminative cleavage of de-esterified pectin. However,  
12 *T. fusca* does not appear to possess a pectin methylesterase or a pectin acetylerase gene.  
13 These observations agree with experimental evidence showing that *T. fusca* cannot grow on  
14 pectin but does grow on its de-esterified derivative, polygalacturonic acid (pectate), which is the  
15 major pectin polymer in the cell walls of most plants. Besides pectate lysases, *T. fusca* has a  
16 gene (Tfu2009) predicted to be a rhamnogalacturonan lyase, which, may be involved in the  
17 degradation of rhamnogalacturonan, a constituent of pectin and pectate.  
18 Besides the glycohydrolases mentioned above, the *T. fusca* genome encodes a set of four CDSs  
19 that possess a CBM domain without any identifiable glycohydrolase catalytic domain. Tfu0644  
20 is predicted to have one predicted transmembrane helix, exposing the CBM2 domain to the  
21 extracellular face of the membrane. Tfu1268 is a secreted protein containing a CBM33 chitin  
22 binding domain. Tfu1665 is a secreted protein that contains two CBMs: a chitin binding domain  
23 at its N-terminus (CBM33) and a C-terminal cellulose binding module (CBM2). We hypothesize  
24 that these proteins probably participate in plant cell wall degradation by providing scaffold



1 services to hydrolytic enzymes and facilitating their action. Tfu1643 is an intracellular protein  
2 with a ricin-type lectin domain which presumably binds and interacts with carbohydrates.

3 **Transcription and translation.** There are 29 large and 17 small ribosomal subunit proteins for  
4 the assembly of the ribosome and the typical prokaryotic translation initiation factors (IF1, IF2,  
5 IF3) and elongation factors (Tu and G) are present. Fifty-three CDSs code for tRNAs covering  
6 all 20 amino acids.

7 The transcriptional regulation of six *T. fusca* cellulase (Tfu0620, Tfu0901, Tfu1074, Tfu1627,  
8 Tfu1959, Tfu2176) genes is mediated by the CelR gene product (Tfu0938) which binds to a 14-  
9 bp inverted repeat (5-TGGGAGCGCTCCCA) in their 5'-upstream regions (44, 45). CelR acts  
10 as a repressor and physiological concentrations of cellobiose (the major end product of  
11 cellulases) cause dissociation of the CelR-DNA complex. The 14-bp inverted repeat mentioned  
12 above is found in additional five locations in the *T. fusca* genome.

13 It is found upstream of Tfu0934 which, together with Tfu935 and Tfu936, constitutes a  
14 disaccharide ABC transport cassette. The chromosomal location of this cassette is immediately  
15 upstream of the intracellular  $\beta$ -glucosidase *bglC*, Tfu0937 (46), and the CelR coding gene  
16 (Tfu0938). Spiridonov et al characterized the operon Tfu935-Tfu938 and postulated that  
17 imperfect palindromes located upstream of Tfu935 may represent putative CelR binding sites.

18 However, the existence of the perfect palindrome 5'-upstream of Tfu934 suggests that the set of  
19 Tfu0934-Tfu0938 may be transcriptionally coregulated by CelR. There are four additional genes  
20 with perfect CelR binding sites at their 5' region: Tfu1135, Tfu1508, Tfu1665, and Tfu2844.

21 Tfu1135 is a unique protein in *T. fusca* with no apparent homologs or similar genes in other  
22 organisms. Tfu1508 is a membrane protein of the major facilitator family and it may be  
23 involved in the transport of the cellulose hydrolysis products into the cell. Tfu1665 is a secreted  
24 protein that contains three carbohydrate binding domains: an N-terminal chitin binding domain, a  
25 C-terminal cellulose binding domain (CBM\_2 class) and a fibronectin type III binding region

1 located in the middle of the protein. This structure suggests that Tfu1665 may act as a scaffold  
2 protein mediating the action of other carbohydrate hydrolyzing enzymes. It does not contain a  
3 TAT signal and presumably it is secreted via the *sec* pathway. Finally, Tfu2844 is an  
4 oxidoreductase of unknown specificity.

5 Besides the above mentioned genes that contain perfect CelR binding palindromes an additional  
6 secreted xylanase (Tfu2923) has an imperfect palindrome in its upstream region (5-  
7 TGGGAGCGCTCCCG). Interestingly, this imperfect palindrome is also located next to the  
8 perfect one at the regulatory regions upstream of Tfu0621 and Tfu1959 suggesting that it may  
9 also participate in the regulation of the expression of these cellulases. The same perfect  
10 palindrome is found in ten locations in the genome of *S. coelicolor* and four locations in the  
11 genome of *S. avermitilis*. In the case of *S. coelicolor* the palindrome is located upstream of six  
12 secreted glycosylhydrolases (SCO0554, SCO0643, SCO0765, SCO1187, SCO6546 and  
13 SCO7637), a protein that contains a cellulose binding domain (SCO5786), and one *lac*-type  
14 transcriptional regulator (SCO2794). In the case of *S. avermitilis* the palindrome is located  
15 upstream of two secreted glycosylhydrolases (SAV555, SAV1854) as well as the cellobiose  
16 transporter (SAV5256, SAV5255, and SAV5254) and a *lac*-type transcriptional regulator  
17 (SAV5257).

18 There are six additional genes (Tfu2790, Tfu1620, Tfu1710, Tfu0834, Tfu1922 and Tfu0909)  
19 that have significant similarity to CelR and belong to the lactose repressor family (*lac*) and they  
20 might be involved in carbohydrate-induced transcriptional regulation. These genes may respond  
21 to different agonists than CelR. CelR is probably not a universal transcriptional regulator since  
22 its levels are diminished when *T. fusca* grows on glucose or xylan. All of the above *lac*-type  
23 CDSs except Tfu1710 are localized on the chromosome adjacent to saccharide transporters and  
24 glycoside hydrolases (Figure 4A). Based on this observation we hypothesize that they regulate  
25 the expression of the respective genomic regions.

1 **Protein transport, secretion, adherence and motility.** *T. fusca* has a complete set of proteins  
2 comprising a *sec* system for general protein secretion. It has homologs of the *secA* (Tfu2490,  
3 Tfu0761), *secY* (Tfu2626), *secE* (Tfu2660), *secG* (Tfu2014) and *yajC* (Tfu2092) secretion  
4 components. A signal peptidase (Tfu0667) is also present. Most organisms have one copy of the  
5 *secA* protein family and recent evidence suggests that the second copy has distinct functions (5).  
6 In *Actinobacteria* a second copy of *secA* exists in *Bifidobacterium longum* DJO10A, all  
7 *Corynebacteria* and *Mycobacteria* sequenced to date, and *S. avermitilis*. *T. fusca* has all the  
8 components of the *sec*-independent twin-arginine protein translocation (TAT) system Tfu1768  
9 (*tatC*), Tfu1769 (*tatA/E*), Tfu0381 (*tatB*), Tfu0398 (*tatD*). The distinguishing feature of the TAT  
10 system is its ability to translocate fully folded proteins and it may have significant implications  
11 for the secretion of functional cellulases. The genome does not contain any homologs of the type  
12 I and type III secretion systems. However, CDSs with similarity to components of the type II  
13 secretion system have been identified. Previous work demonstrated that the secretion of a Cel5  
14 cellulase by *Erwinia chrysanthemi* was carried out by a type II protein secretion system (6). On  
15 the contrary, Faury *et al* reported that the TAT export system mediates the secretion of an active  
16 xylanase from *Streptomyces lividans* (9). Moreover, recent work demonstrated that the TAT  
17 system is the major route for protein export in *S. coelicolor* (52). Therefore, we investigated  
18 whether the *T. fusca* genome encodes components of the type II secretion apparatus. Type II  
19 secretion systems generally consist of 12 proteins and share many components with the type IV  
20 pilus biogenesis machinery (35). Examination of the genome revealed the existence of CDSs  
21 similar to type II and IV secretion components distributed between two operons. The first  
22 operon contained three CDSs with homologies to pilus assembly protein TadB (Tfu0126,  
23 Tfu0127) and the cytoplasmic ATPase of the *pilE* type (Tfu0128).  
24 The second operon, located between Tfu2271 and Tfu2276, contains proteins with homologies to  
25 the pilus assembly proteins CpaB (Tfu2271), TadG (Tfu2273), GSP<sub>II</sub>\_F (Tfu2275, Tfu2276). It

1 also contains two predicted ATPases, Tfu2274 which contains a pulE like domain (Pfam  
2 GSPII\_E) and Tfu2272 which is also an ATPase containing a receiver domain at its amino  
3 terminal region. However, we were unable to identify any homologs of the secretin (T2SD),  
4 which is the component forming channels for protein efflux, in *T. fusca* and other  
5 *Actinobacteria*. On the contrary, the gene similarities and structure of the respective  
6 chromosomal regions resemble the *tight adherence (tad)* locus (Fig. 4B) identified in the gram-  
7 negative bacterium *Actinobacillus actinomycetemcomitans* (20, 37, 42). The *tad* locus has been  
8 identified as an important mechanism for the production of pili that mediate nonspecific  
9 adherence to surfaces and formation of biofilms (21). However, both *T. fusca* and *S. coelicolor*  
10 lack homologs of either *rcpA* (a secretin family protein) or prepilins. We hypothesize that these  
11 particular functions have been substituted by non-orthologous genes in *T. fusca*. Overall, based  
12 on the above observations we propose that *T. fusca* lacks a type II secretion system and the above  
13 mentioned CDSs constitute a system for non-specific adherence to surfaces analogous to the one  
14 characterized in *Actinobacillus actinomycetemcomitans* (36, 37). Consequently, we  
15 hypothesize that the secretion of cellulases in *T. fusca* is mediated by the TAT translocation  
16 system and the general *sec* secretion system.

17 **Transport systems:** Approximately 6% (180 CDSs) of the *T. fusca* genome encodes genes for  
18 transport. *T. fusca* has the core phosphotransferase system components E1 (Tfu2489) and Hpr  
19 (Tfu2487) as do all published *Actinobacteria* except *Tropheryma* (reduced genome) and  
20 *Mycobacteria*. However, unlike other *Actinobacteria* it has no identifiable homologs of PTS  
21 transporters. Therefore, we hypothesize that *T. fusca* does not have an active PTS transport  
22 system and the E1 and Hpr components function in signaling pathways. This is a major  
23 difference with the carbohydrate transport system of *S. coelicolor* which utilizes the PTS system  
24 to transport fructose and N-acetylglucosamine (33, 34).

1 *T. fusca* has an extensive set of transporters for carbohydrate uptake. Eight ABC disaccharide  
2 transporter cassettes were identified in the genome and the majority of them (5) are localized on  
3 the chromosome next to glycoside hydrolases (Fig. 4A). The transport system (Tfu0934-  
4 Tfu0936) that clusters with the CelR transcription factor (see above) exhibits high similarity to  
5 the cellobiose/cellotriose transport system of *Streptomyces reticuli* (40). This system lacks an  
6 ATPase component in both organisms. However, *T. fusca* harbors a homolog of the MsiK  
7 (multiple sugar import protein) protein, Tfu1763. MsiK has been characterized in *Streptomyces*  
8 and it was shown to assist both in cellobiose and maltose transport (41). The *T. fusca* maltose  
9 transport system consists of three CDSs, Tfu0830, Tfu0831, and Tfu0832. It is clustered on the  
10 chromosome with Tfu0833 which is an intracellular 1,4- $\alpha$ -D-glucan glucanohydrolase, probably  
11 involved in the hydrolysis of maltose. The adjacent CDS (Tfu0834) is a *lacI*-type transcriptional  
12 regulation and may be involved in the transcription of the transporter-glycohydrolase operon.  
13 The chromosomal region between Tfu1609 and Tfu1620 (12 CDSs) contains two transport  
14 systems, a set of two secreted and three intracellular glycosyl hydrolases as well as a *lac*-type  
15 transcriptional regulator (Fig 4A). Tfu1617, Tfu1618 and Tfu1619 are components of the  
16 xylobiose transporter based on their high similarity to the recently characterized xylobiose  
17 transporter from *Streptomyces thermoviolaceus* (49). Tfu1612 is the secreted xyloglucanase  
18 possessing a potential TAT targeting signal peptide characterized by Irwin et al (17). Tfu1616 is  
19 a GH\_43 family protein encoding an uncharacterized secreted glycoside hydrolase activity,  
20 whereas Tfu1613, Tfu1614 and Tfu1615 are intracellular glycoside hydrolases. Tfu1609,  
21 Tfu1610 and Tfu1611 are components of a putative disaccharide transport system with unknown  
22 specificity. Tfu1620 is a *lac*-type transcriptional regulator and we postulate that it regulates the  
23 expression of the gene set Tfu1609-Tfu1620.

24 The genome of *T. fusca* also has an ortholog (Tfu1668) of the *glcP* sugar permease characterized  
25 in *S. coelicolor* as a major glucose uptake system (51). Overall, our analysis identified the

1 presence of cellobiose/cellotriose, maltose and xylobiose ABC transport systems, and a permease  
2 of the major facilitator superfamily for glucose uptake.

3 There are four putative amino acid ABC transport systems (Tfu0304-Tfu0306, Tfu0910-  
4 Tfu0914, Tfu1182-Tfu1187, and Tfu2928-Tfu2930).

5 Genes encoding a siderophore transport system were identified (Tfu0656, Tfu0657, Tfu1491-  
6 Tfu1494)) as well as a siderophore biosynthesis cluster (Tfu1865-Tfu1867). In addition there is  
7 a ferrous iron transport protein (Tfu0820). There are four additional heavy metal transport  
8 systems (Tfu0857-Tfu0858, Tfu0931-Tfu0932, Tfu1661-Tfu1663, Tfu2312-Tfu2313).

9 **DNA repair and replication, stress.** As compared to many other representatives of  
10 *Actinobacteria*, especially some pathogenic species, *T. fusca* has reduced DNA repair  
11 capabilities and fewer mechanism of resistance to reactive oxygen species (Table 1). *T. fusca* has  
12 Mn-dependent and heme catalases (Tfu0886 and Tfu1649, respectively), and Mn-dependent  
13 superoxide dismutase (Tfu0957), while other enzymes present in *N. farcinica*, *Streptomyces spp.*  
14 and *Mycobacterium spp.*, such as catalase/peroxidase and Cu-Zn superoxide dismutase are  
15 missing. Similarly, only a homolog of the organic hydroperoxide resistance protein Ohr  
16 (Tfu0697) is present (8), while peroxiredoxin reductase AhpD and peroxiredoxin AhpC are  
17 absent.

18 *T. fusca* is capable of repairing UV-induced cyclobutane pyrimidine dimers due to the  
19 presence of type I deoxyribopyrimidine photolyase (Tfu0534). Deaminated bases are removed  
20 via base excision mechanism (uracil-DNA glycosylase Tfu1341 and G/U-mismatch DNA  
21 glycosylase Tfu1918), while oxidized bases are removed by endonuclease III (Tfu0118),  
22 formamidopyrimidine-DNA glycosylase (Tfu0652) and A/G-specific adenine glycosylase  
23 (Tfu2875) and the resulting abasic sites are processed by the DNA-(apurinic or apyrimidinic site)  
24 lyase activity of some these enzymes or by endonuclease IV (Tfu1956) and endonuclease V  
25 (Tfu1400). The repertoire of pathways for repair of alkylated bases is limited in *T. fusca* to base

1 excision by DNA-3-methyladenine glycosylase I (Tfu0498) and alkylation reversal via DNA-  
2 N1-methyladenine dioxygenase (Tfu1427) (23); no homologs of other methyladenine  
3 glycosylases or a suicidal protein-cysteine S-methyltransferase AidA were found. Genes coding  
4 for the subunits of excinuclease ABC, an enzyme responsible for nucleotide excision repair are  
5 also present in the genome (Tfu2024, Tfu1196 and Tfu2021). Similar to other *Actinobacteria*,  
6 the genes coding for methyl-directed mismatch repair system (MutSLH proteins) is absent. *T.*  
7 *fusca* has only one error-prone DNA polymerase responsible for translesion synthesis (Tfu1096).  
8 Unlike many other representatives of *Actinobacteria*, no homologs of proteins responsible for  
9 double-strand break repair by non-homologous end joining [3] were found in the genome of *T.*  
10 *fusca* (13).

11         One of the most striking features of *T. fusca* is the genetic organization of the *recA* gene  
12 (Tfu0803), which has two inteins (see NEB Intein Database,  
13 <http://www.neb.com/neb/inteins.html>) inserted at both the RecA-a and RecA-b sites [4]. While  
14 intein-harboring *recA* genes appear to be quite common among both pathogenic and free-living  
15 mycobacteria (39), none of the genes sequenced so far has two intein insertions. Since it has been  
16 demonstrated that the intein-free RecA protein from *Mycobacterium smegmatis* and single  
17 intein-containing RecA protein from *Mycobacterium tuberculosis* display different activity in  
18 DNA strand exchange (10), RecA from *T. fusca* represents an interesting model to further clarify  
19 the mechanistic basis and the factors that contribute to the extent of DNA strand transfer in  
20 various *Actinobacteria*. Other features of recombinational DNA repair include the absence of a  
21 RecBCD complex or its AddAB equivalent with DNA helicase and exonuclease activities. Some  
22 of the essential components of an alternative RecF recombination pathway operating in *recBC*  
23 mutants in *E. coli* (43) are also missing; these include helicase RecQ and 5'-3' exonuclease RecJ.  
24 However, despite the apparent absence of any helicase or exonuclease activity typically  
25 associated with DNA recombination, other components of a recombination apparatus (24), such

1 as RecF, RecO, RecR and RecN proteins (Tfu0004, Tfu0852, Tfu0044 and Tfu2032,  
2 respectively) and enzymes responsible for Holliday junction resolution (Tfu2093, Tfu2094,  
3 Tfu2095 and Tfu0646) are present in the genome. Further analysis of phylogenetic distribution  
4 of the genes coding for various components of recombination machinery in *Actinobacteria*  
5 reveals no correlation between the genetic structure of the *recA* gene and the presence of the  
6 proteins involved in RecBCD or RecF recombination pathways.

7 **Central metabolism.** The *T. fusca* genome encodes all the enzymes necessary to carry out  
8 glycolytic degradation of monosaccharides. Additionally, it has the Entner-Doudoroff pathway  
9 for glucose utilization.

10 All the enzymes of TCA cycle are present. The presence of the glyoxylate cycle-bypass from  
11 isocitrate to malate is indicative of its ability to grow on additional carbon sources such as  
12 acetate. The existence of homologs of glycerol kinase (Tfu0787), glycerol-3-phosphate  
13 dehydrogenase (Tfu0631) and triose phosphate isomerase (Tfu2015) indicates its ability to  
14 convert glycerol to glyceraldehyde-3-phosphate, an intermediate of glycolysis. Therefore,  
15 glycerol can also serve as a carbon source. It has the gluconeogenic enzymes pyruvate  
16 carboxylase (Tfu2554), phosphoenolpyruvate carboxykinase (Tfu0083) and fructose 1,6  
17 biphosphatase (Tfu0464) indicative of gluconeogenesis.

18 It has the machinery for the *de novo* biosynthesis of all amino acids except asparagine.  
19 However, it possesses a class II aspartyl-tRNA synthetase (Tfu2086) indicative of its ability to  
20 synthesize Asn-tRNA through transamidation of Asp-tRNA (32).

21 All the enzymes necessary for the biosynthesis of purines and pyrimidines have been identified.

22 *T. fusca* has the enzymes for NAD, CoA, riboflavin, pyridoxal, folate, cobalamin and  
23 menaquinone biosynthesis. Two pathways have been described for the biosynthesis of  
24 quinolinate, an intermediate in NAD biosynthesis (26). The tryptophan to quinolinate pathway is  
25 present in *T. fusca* as well as in both sequenced *Streptomyces* strains sequenced until now, but it



1 is absent in the rest of the *Actinobacteria*. *T. fusca* also utilizes the aspartate to quinolinate  
2 pathway which is common to all *Actinobacteria*. *T. fusca* does not have a biotin pathway  
3 biosynthesis since homologs of bioB, bioF and bioD have not been identified. However, it  
4 possesses a transporter (Tfu2314) for scavenging biotin from the environment. The biosynthetic  
5 pathway of thiamin is also incomplete.

6 **Lipid and cell wall metabolism.** *T. fusca* is able to synthesize and metabolize saturated and  
7 unsaturated fatty acids, as well as other major lipid classes such as phospholipids, glycolipids  
8 and isoprenoids. *T. fusca* appears to have all the enzymes necessary for fatty acid biosynthesis.  
9 It utilizes exclusively a type II fatty acid synthesis system in common with *Streptomyces* and in  
10 contrast to *Mycobacteria*, *Corynebacteria* and *Nocardia* which utilize a type I fatty acid synthase  
11 in addition to the type II system. It generates unsaturated fatty acids by a fatty acid desaturase  
12 mechanism (Tfu0413). An interesting feature of the *T. fusca* genome lies in the existence of a  
13 PlsX homolog in the absence of its PlsY counterpart. PlsX and PlsY were recently shown to  
14 constitute a novel mechanism for phospholipid biosynthesis initiation through the formation of  
15 acylphosphates (30). *T. fusca* appears to lack both a PlsB as well as a PlsY homolog raising the  
16 intriguing possibility that an as yet unidentified enzyme participates in the initiation of  
17 phospholipid biosynthesis.

18 Two secreted triacylglycerol lipases can be identified in the genome (Tfu0882, Tfu0883). It has  
19 the enzymes for  $\beta$ -oxidation of both odd and even carbon number fatty acids. In contrast to  
20 *Mycobacteria* and *Streptomyces* it does not have homologs of diacylglycerol acyltransferases  
21 and, therefore, it probably lacks the machinery for endocellular triacylglycerol accumulation.  
22 Hence, these observations suggest that *T. fusca* can hydrolyse extracellular lipids, take up the  
23 resulting fatty acids and utilize them subsequently as a carbon source. *T. fusca* has all the genes  
24 for the biosynthesis of phosphatidylglycerol, phosphatidylserine, phosphatidylethanolamine and  
25 phosphatidylinositol. Phosphatidylinositol is further decorated with mannosyl-groups as

1 evidenced by the existence of a phosphatidylinositol mannosyltransferase homolog (Tfu2101). It  
2 utilizes a phospholipase D-type of cardiolipin synthase (Tfu2817). This type of enzyme catalyzes  
3 the synthesis of cardiolipin from two molecules of phosphatidylglycerol. In contrast,  
4 *Mycobacteria* and *Nocardia* appear to possess a “eukaryotic” type reaction via a CDP-alcohol  
5 phosphatidyltransferase which catalyzes cardiolipin formation from CDP-diacylglycerol and  
6 phosphatidylglycerol.

7 Additionally, *T. fusca* and both *S. coelicolor* and *S. avermitilis* have two novel classes of CDP-  
8 alcohol phosphatidyltransferases (Tfu2439, Tfu2359) indicative of their ability to synthesize as  
9 yet unidentified phospholipids. The most attractive hypothesis is that these enzymes catalyze the  
10 formation of novel structures of phosphatidylsugars.

11 It possesses a complete non-mevalonate pathway for isoprenoid biosynthesis. The existence of  
12 genes with homology to phytoene synthase (Tfu3076), phytoene dehydrogenase (Tfu3075) and  
13 lycopene cyclase (Tfu3088) is indicative of a putative carotenogenesis process (Fig. 4C). A  
14 recent study established the ability of *S. coelicolor* to carry out light induced synthesis of  
15 carotenoids (48).

16 All the genes (*murC*, *murD*, *murE* and *murF*) that encode enzymes for the conversion of D-  
17 glutamate to UDP-MurNac pentapeptide are present, as are other enzymes in the peptidoglycan  
18 synthetic pathway such as GlmU, MurA and MurB.

19 **Conclusions.** The genome sequence of *T. fusca* provides the means for a detailed analysis of the  
20 cellular mechanisms controlling the expression and secretion of plant cell-wall degrading  
21 enzymes by this soil bacterium. *T. fusca* utilizes a variety of enzymes attacking cellulose, xylan  
22 and pectin, major components of plant cell walls. Detailed genomic analysis provides evidence  
23 for the utilization of the TAT secretion system for the export of these enzymes to extracellular  
24 space, the existence of multiple transcription factors regulating the expression of  
25 glycosylhydrolases and oligo/poly saccharide transport systems. These observations open

1 further research directions for understanding the mechanisms of plant cell wall hydrolysis and  
2 utilization by soil actinomycetes.

### 3 **ACKNOWLEDGEMENTS**

4 This study was performed under the auspices of the U.S. Department of Energy's Office of  
5 Science, Biological and Environmental Research Program, and by the University of California,  
6 Lawrence Berkeley National Laboratory under contract DE-AC02-05CH11231, and Lawrence  
7 Livermore National Laboratory, under contract DE-AC02-06NA25396.

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23 **Figure legends.**

24 Figure 1. Circular representation of the genome of *T. fusca*. From outside to inside, the first two  
25 circles represent COG assignment for predicted coding sequences on the plus and minus  
26 strands, respectively. Colors indicate the following: dark gray, hypothetical proteins;  
27 light gray, conserved hypothetical and unknown function; brown, general function  
28 prediction; red, replication and repair; green, energy metabolism; blue, carbon and  
29 carbohydrate metabolism; cyan, lipid metabolism; magenta, transcription; yellow,  
30 translation; orange, amino acid metabolism; pink, metabolism of cofactors and vitamins;  
31 light red, purine and pyrimidine metabolism; lavender, signal transduction; and blue sky,  
32 cellular processes. The two innermost circles represent the %G+C content and G+C  
33 skew values, respectively.

34 Figure 2. Amino acid utilization in *T. fusca*. Comprison of the percentage content of amino acid  
35 residues in all sequenced Bacteria, *Actinobacteria* and *T. fusca*.

1 Figure 3. Synteny plots between *T. fusca* (horizontal axis) and five representative genomes of  
2 *Actinobacteria*: *Streptomyces coelicolor*, *Nocardia farcinica*, *Mycobacterium*  
3 *tuberculosis*, and *Corynebacterium diphtheria*.

4 Figure 4. Organization of *T. fusca* chromosomal regions. Each arrow represents one gene.  
5 Locus tags and putative gene names are indicated. Tf, *T. fusca*; Aa, *A.*  
6 *actinomycetemcomitans*; Sc, *S. Coelicolor*; Sa, *S. Avermitilis*. **A.** Chromosomal  
7 clustering of *T. fusca* secreted and intracellular glycoside hydrolases with carbohydrate  
8 transporters and *lac*-type transcription factors. **B.** Comparison of the tight adherence  
9 locus of *A. actinomycetemcomitans* to the chromosomal regions of *T. fusca* and *S.*  
10 *coelicolor* that contain homologous genes. **C.** Comparison of the *S. coelicolor* cluster  
11 encoding genes predicted to participate in carotene biosynthesis to the locus of  
12 homologous genes in *S. avermitilis* and *T. fusca*.

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2 Table 1. Global genomic comparison among five representatives of *Actinobacteria*. Top 10 lines  
3 depict unique genes: Number of genes in the row organism without a homolog in the column  
4 organism; Lines 7-10, common genes in the row organism pair without homolog in the column  
5 organism. Bottom five lines, common genes: Number of genes in the column organism with a  
6 homolog in the row organism. Tf, *T. fusca*; Sc, *S. coelicolor*; Mt, *M. tuberculosis* H37Rv; Cd, *C.*  
7 *diphtheriae* NCTC 13129; Nf, *N. farcinica* IFM 10152. The calculations were based on a  
8 maximum *E*-value of  $10^{-3}$  and minimum identity 30%.

9

	<i>Tf</i>	<i>Sc</i>	<i>Mt</i>	<i>Cd</i>	<i>Nf</i>
<i>Tf</i>		838	1436	1840	1077
<i>Sc</i>	4310		5038	6125	4007
<i>Mt</i>	2184	1764		2548	1413
<i>Cd</i>	1191	1058	1076		921
<i>Nf</i>	3196	2495	2894	4109	
<i>Tf+Sc</i>			673	1055	409
<i>Tf+Mt</i>		75		555	85
<i>Tf+Cd</i>		53	151		51
<i>Tf+Nf</i>		170	444	814	
<i>Tf</i>		2279	1681	1277	2040
<i>Sc</i>	3905		3177	2090	4208
<i>Mt</i>	1815	2235		1451	2586
<i>Cd</i>	1129	1262	1244		1399
<i>Nf</i>	2740	3441	3042	1827	

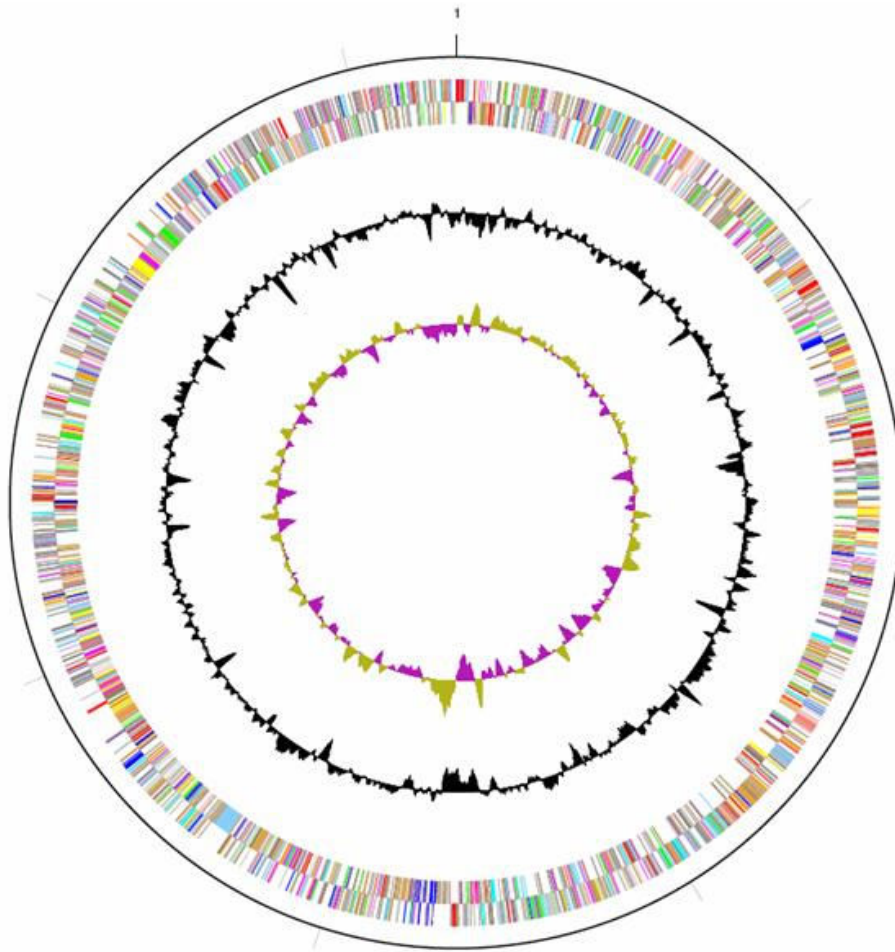


Table 2. Summary of glycoside hydrolases and CBM containing proteins in *T. fusca*

Locus tag	Family	Description	Binding module	signal peptide	Potential TAT signal	Ref
Tfu0082	CE_1	acetyl xylan esterase		Yes	Yes,RRIPARVWVALTAVLGLGAALLGTTALAPRAEAA	
Tfu2990	CE_3	acetyl xylan esterase	Cellulose, CBM2	Yes	No	
Tfu1621	CE_4	acetyl xylan esterase/chitin deacetylase	LBP	Yes	No	
Tfu2788	CE_4	acetyl xylan esterase/chitin deacetylase	Cellulose, CBM2	Yes	Yes, PRRSPLRKRLLVALCALGLAFTSAATAHAQV	
Tfu2789	CE_4	acetyl xylan esterase/chitin deacetylase		Yes	No	
Tfu2473	CE_9	6P-NAG deacetylase/6P-NAGal deacetylase		No		
Tfu0084	CE_14	GlcNAc-PI deacetylase		No		
Tfu0449	CE_14	GlcNAc-PI deacetylase		No		
Tfu0486	CE_14	GlcNAc-PI deacetylase		No		
Tfu0937	GH_1	<b>beta glucosidase/ cellobiase</b>		No		(46)
Tfu1629	GH_1	glucosidase/galactosidase		Yes	No	
Tfu0915	GH_2	galactosidase		No		
Tfu1607	GH_3	xylosidase/acetlyhexosaminidase	$\beta$ -1,3-glucan, CBM6	No		
Tfu2486	GH_3	xylosidase/acetlyhexosaminidase		Yes		
Tfu2768	GH_4	glucuronidase/galactosidase/glucosidase		No		
Tfu0900	GH_5	<b>beta-mannanase</b> (EC 3.2.1.78)	Cellulose, CBM2	Yes	Yes, RKRLAVAAATVLALLASVFALTQPANAAT	(14)
Tfu0901	GH_5	<b>endocellulase (E5)</b>	Cellulose, CBM2	Yes	Yes, RKGPPVAVAVTAALALLIALLSPGVAQAAG	(28)
Tfu2712	GH_5	<b>Cellulase</b>	Cellulose, CBM3	Yes	Yes, RRLRAGAAAIAIGASALIPLTSSPAAASG	(38)
Tfu0620	GH_6	<b>beta 1,4 exocellulase (E3)</b>	Cellulose, CBM2	Yes	Yes, RRSWMRRGLAAASG	(28, 53)
Tfu1074	GH_6	<b>beta 1,4 endoglucanase (E2)</b>	Cellulose, CBM2	Yes	No	(28)
Tfu1627	GH_9	<b>beta-1,4 endoglucanase (E1)</b>	Cellulose/amorphous/xylan, CBM2, CBM4	Yes	Yes, RRRSRSPPLVAL	(19)
Tfu2176	GH_9	<b>Cellulase (E4)</b>	Cellulose, CBM2, CBM3	Yes	Yes, PRRRGRHSRARRF	(19, 28)
Tfu2791	GH_10	<b>Xylanase</b>		Yes	No	(22)
Tfu2923	GH_10	<b>Xylanase</b>	Cellulose, CBM2	Yes	Yes, RHRPSRRARRSLLLTSALTAAGLLVTAAPAQAES	(4)
Tfu1213	GH_11	<b>endo 1,4 beta D xylanase</b>	Cellulose, CBM2	Yes	Yes, RRRFRPRLIGKAFAAALVAVVTMIPSTAHAHAV	(16)
Tfu0582	GH_13	amylase/pullulanase		No		
Tfu0584	GH_13	amylase		No		
Tfu0585	GH_13	amylase/pullulanase		No		
Tfu0833	GH_13	amylase/pullulanase		No		
Tfu0985	GH_13	amylase/pullulanase	Starch, CBM20	Yes	Yes, RRSLAALLAALLGCATSLVALTVAASPAHAAP	
Tfu1891	GH_13	amylase/pullulanase		No		
Tfu0046	GH_15	amylase/dextranase		No		

Tfu1345	GH_15	amylase/dextranase		No		
Tfu0580	GH_18	chitinase		No		
Tfu0868	GH_18	exochitinase		Yes	Yes, RRRTFAPTWWVLLVAAGWALC	
Tfu0480	GH_23	lysozyme		No		
Tfu2594	GH_23	lysozyme		No		
Tfu1613	GH_31	amylase/isomaltase		No		
Tfu1615	GH_42	galactosidase		No		
Tfu1616	GH_43	xylanase/galactan galactosidase		Yes		
Tfu1959	GH_48	<b>beta 1,4 exocellulase (E6-celF)</b>	Cellulose, CBM2	Yes	Yes, RRWRTLASGALAAALAAVLSPGVAHAAV	(18)
Tfu3044	GH_65	trehalase/maltose phosphorylase		No		
Tfu1612	GH_74	<b>Xyloglucanase, endo-β-1,4-glucanase</b>	Cellulose, CBM2	Yes	Yes, RRRGIARALTCIAAAATVAAVG	(17)
Tfu2205	GH_77	amylomaltase or 4--glucanotransferase		No		
Tfu2130	GH_81	<b>Glucanase</b>		Yes	Yes, RRRWRRATTSAAATAALLCGALLTFPSAPAAA	(31)
Tfu1614	GH_95	fucosidase		No		
Tfu0153	PL_1	pectate_lyase (EC 4.2.2.2)		Yes	Yes, MRRAATLGVALALPLTLAAPSSALAQP	
Tfu2168	PL_1	pectate_lyase (EC 4.2.2.2)		Yes	Yes, VGRSITRRLASTLATAAVVTAGLTLPVSPAQAQT	
Tfu0644			Cellulose, CBM2	No		
Tfu1268			Chitin, CBM33	Yes		
Tfu1643			Xylan/mannose/galactose, CBM13	No	No	
Tfu1665			cellulose/chitin, CBM2, CBM33	Yes	No	
Tfu2009		rhamnogalacturonan lyase	Cellulose, CBM2	Yes	Yes, RRRPVRFGAALAAFVLGATGAAALPSPAHAHA	

Figure 1



Total bases	3,642,249	Protein coding genes	3117
DNA coding bases	3,122,831 (85.74%)	With predicted function	2181 (68.54%)
GC%	67.5%	Without predicted function	936 (29.40%)
tRNA	53	Pseudogenes	7
5S/16S/23S	4/4/4		

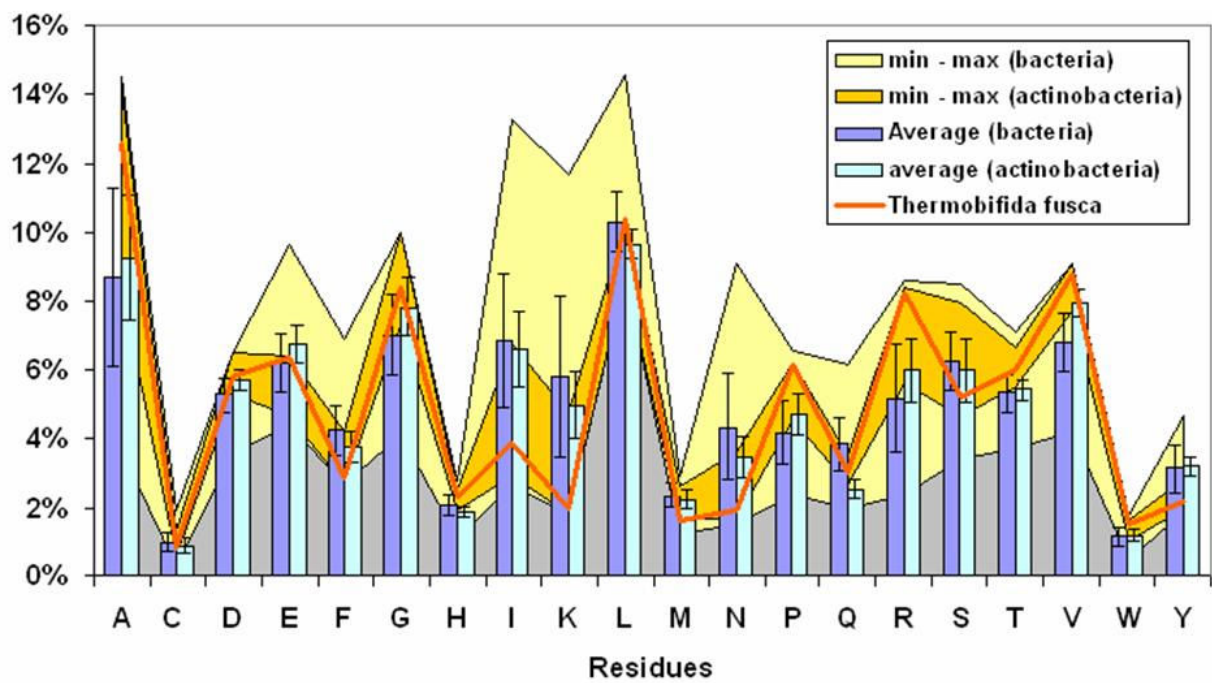
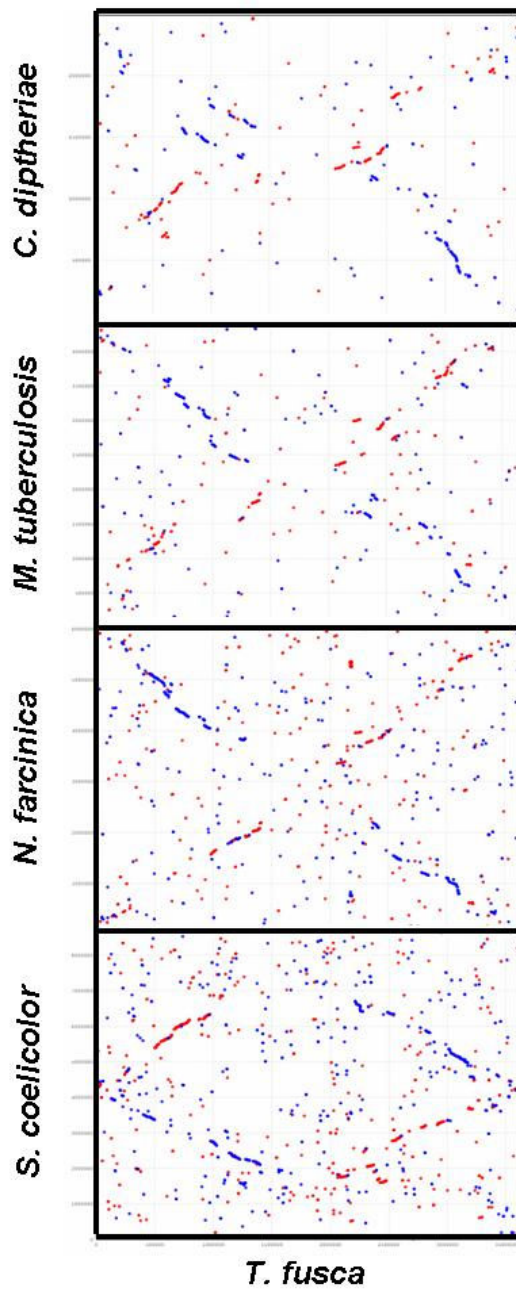


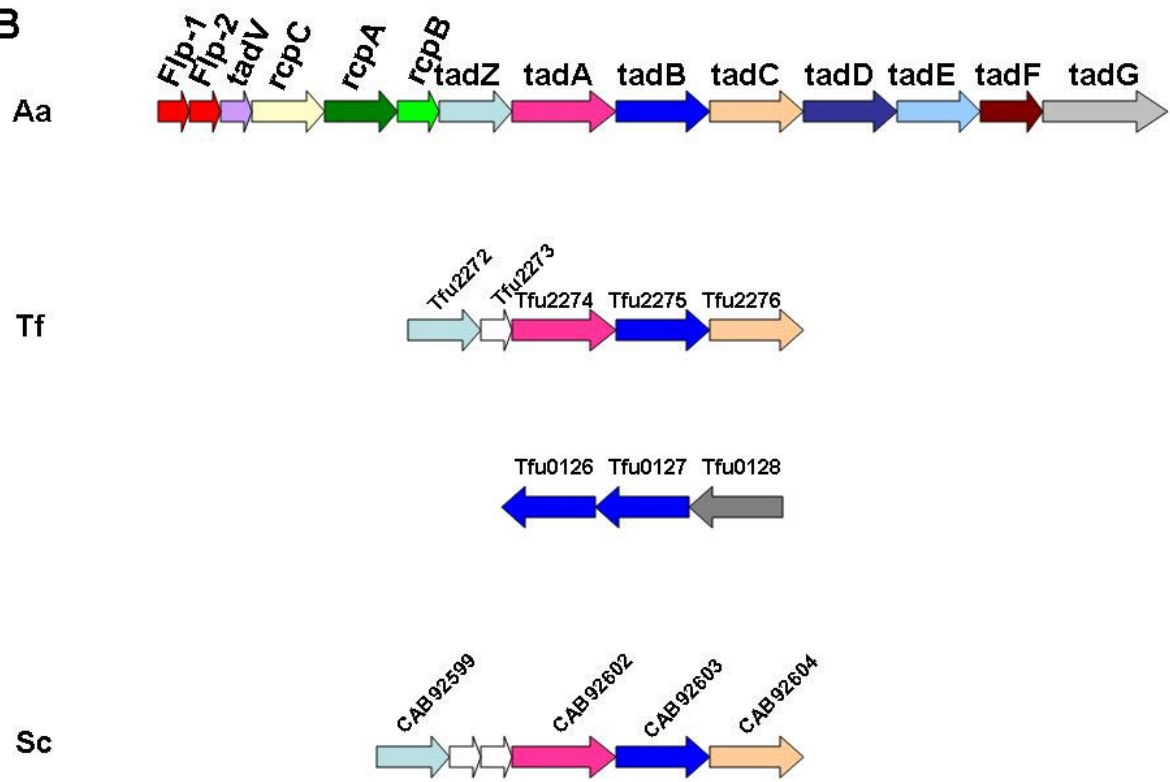
Figure 2

Figure 3





**B**



C

