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

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ABSTRACT

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

INTRODUCTION.

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

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

MATERIALS AND METHODS.

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

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

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

RESULTS AND DISCUSSION.

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

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

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

Cellulases and glycoside hydrolases. The genome of *T. fusca* encodes a total of 45 hydrolytic enzymes predicted to act on oligo- and/or poly-saccharides as identified by the CAZy ModO database, http://afmb.cnrs-mrs.fr/CAZY/ (Table 2). These enzymes include 36 glycoside hydrolases, 9 carbohydrate esterases and 2 polysacharide lyases. The glycoside hydrolases are distributed in 22 families (GH families), with the GH_13 family being the most abundant with six individual members represented in the genome. Computational analysis of signal peptides predicts 16 glycoside hydrolases are secreted. The majority of these secreted proteins (13 out of 16) have a signal sequence resembling the twin-arginine translocation (TAT) signal in their N-terminus. Analysis of the secretion systems will be presented in a subsequent section.

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

similar to endochitinase and exochitinase (Tfu0580 and Tfu0868 respectively). The absence of N-acetyl-hexosaminidase implies that chitin degradation proceeds either with an alternative pathway or by gradual degradation of the substrate by an exochitinolytic enzyme. Deacetylases of chitin, xylan and N-acetyl-glucosamine were identified suggesting that the organism can utilize these substrates for energy production. *T. fusca* contains several enzymes involved in the degradation of xylan: endo-β-1,4-xylanase (Tfu1213,Tfu2791,Tfu2923); xylosidase, (Tfu1613); α-L-arabinofuranosidase (Tfu1616); and several CDSs with putative acetylxylan esterase activity. In addition to the above mentioned carbohydrases *T. fusca* possesses two CDSs with significant similarity to pectate lyases, Tfu0153 and Tfu2168 (Table 2). These two genes encode secreted proteins that possess a signal peptide that may be interacting with the TAT system. Pectate lyases (EC 4.2.2.2) catalyze the eliminative cleavage of de-esterified pectin. However, *T. fusca* does not appear to possess a pectin methylesterase or a pectin acetylesterase gene. These observations agree with experimental evidence showing that *T. fusca* cannot grow on pectin but does grow on its de-esterifed derivitive, polygalacturonic acid (pectate), which is the major pectin polymer in the cell walls of most plants. Besides pectate lysases, *T. fusca* has a gene (Tfu2009) predicted to be a rhamnogalacturonan lyase, which, may be involved in the degradation of rhamnogalacturonan, a constituent of pectin and pectate.

Besides the glycohydrolases mentioned above, the *T. fusca* genome encodes a set of four CDSs that possess a CBM domain without any identifiable glycohydrolase catalytic domain. Tfu0644 is predicted to have one predicted transmembrane helix, exposing the CBM2 domain to the extracellular face of the membrane. Tfu1268 is a secreted protein containing a CBM33 chitin binding domain. Tfu1665 is a secreted protein that contains two CBMs: a chitin binding domain at its N-terminus (CBM33) and a C-terminal cellulose binding module (CBM2). We hypothesize that these proteins probably participate in plant cell wall degradation by providing scaffold

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

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

The transcriptional regulation of six *T. fusca* cellulase (Tfu0620, Tfu0901, Tfu1074, Tfu1627, Tfu1959, Tfu2176) genes is mediated by the CelR gene product (Tfu0938) which binds to a 14-bp inverted repeat (5-TGGGAGCGCTCCCA) in their 5`-upstream regions (44, 45). CelR acts as a repressor and physiological concentrations of cellobiose (the major end product of cellulases) cause dissociation of the CelR-DNA complex. The 14-bp inverted repeat mentioned above is found in additional five locations in the *T. fusca* genome.

It is found upstream of Tfu0934 which, together with Tfu935 and Tfu936, constitutes a disaccharide ABC transport cassette. The chromosomal location of this cassette is immediately upstream of the intracellular β-glucosidace *bglC*, Tfu0937 (46), and the CelR coding gene (Tfu0938). Spiridonov et al characterized the operon Tfu935-Tfu938 and postulated that imperfect palindromes located upstream of Tfu935 may represent putative CelR binding sites. However, the existence of the perfect palindrome 5'-upstream of Tfu934 suggests that the set of Tfu0934-Tfu0938 may be transcriptionally coregulated by CelR. There are four additional genes with perfect CelR binding sites at their 5' region: Tfu1135, Tfu1508, Tfu1665, and Tfu2844. Tfu1135 is a unique protein in *T. fusca* with no apparent homologs or similar genes in other organisms. Tfu1508 is a membrane protein of the major facilitator family and it may be involved in the transport of the cellulose hydrolysis products into the cell. Tfu1665 is a secreted protein that contains three carbohydrate binding domains: an N-terminal chitin binding domain, a C-terminal cellulose binding domain (CBM_2 class) and a fibronectin type III binding region

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

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

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

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

The second operon, located between Tfu2271 and Tfu2276, contains proteins with homologies to the pilus assembly proteins CpaB (Tfu2271), TadG (Tfu2273), GSPII_F (Tfu2275, Tfu2276). It

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

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

T. fusca has an extensive set of transporters for carbohydrate uptake. Eight ABC disaccharide transporter cassettes were identified in the genome and the majority of them (5) are localized on the chromosome next to glycoside hydrolases (Fig. 4A). The transport system (Tfu0934-Tfu0936) that clusters with the CelR transcription factor (see above) exhibits high similarity to the cellobiose/cellotriose transport system of *Streptomyces reticuli* (40). This system lacks an ATPase component in both organisms. However, *T. fusca* harbors a homolog of the MsiK (multiple sugar import protein) protein, Tfu1763. MsiK has been characterized in *Streptomyces* and it was shown to assist both in cellobiose and maltose transport (41). The *T. fusca* maltose transport system consists of three CDSs, Tfu0830, Tfu0831, and Tfu0832. It is clustered on the chromosome with Tfu0833 which is an intracellular 1,4-α-D-glucan glucanohydrolase, probably involved in the hydrolysis of maltose. The adjacent CDS (Tfu0834) is a *lac*I-type transcriptional regulation and may be involved in the transcription of the transporter-glycohydrolase operon. The chromosomal region between Tfu1609 and Tfu1620 (12 CDSs) contains two transport systems, a set of two secreted and three intracellular glycosyl hydrolases as well as a *lac*-type transcriptional regulator (Fig 4A). Tfu1617, Tfu1618 and Tfu1619 are components of the xylobiose transporter based on their high similarity to the recently characterized xylobiose transporter from *Streptomyces thermoviolaceus* (49). Tfu1612 is the secreted xyloglucanase possessing a potential TAT targeting signal peptide characterized by Irwin et al (17). Tfu1616 is a GH_43 family protein encoding an uncharacterized secreted glycoside hydrolase activity, whereas Tfu1613, Tfu1614 and Tfu1615 are intracellular glycoside hydrolases. Tfu1609, Tfu1610 and Tfu1611 are components of a putative disaccharide transport system with unknown specificity. Tfu1620 is a *lac*-type transcriptional regulator and we postulate that it regulates the expression of the gene set Tfu1609-Tfu1620.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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Figure legends.

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

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

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

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

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

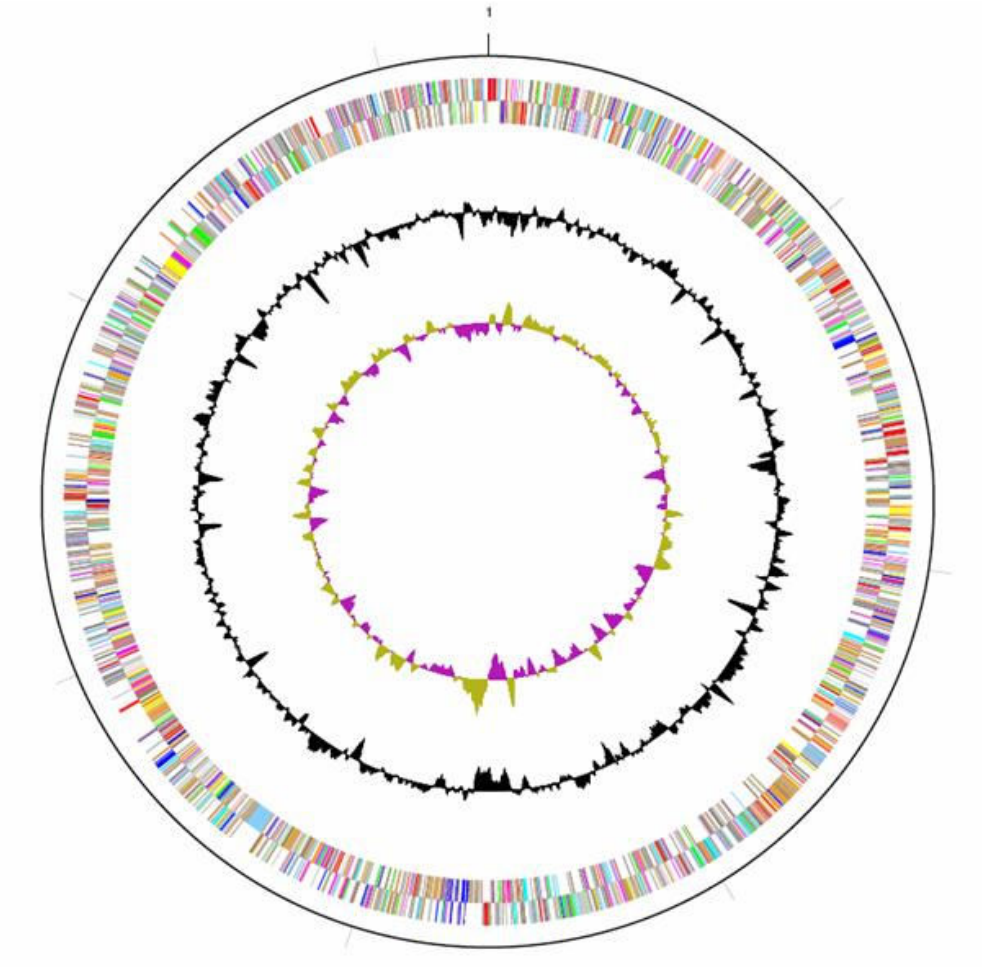
	Tf	*Sc*	*Mt*	*Cd*	*Nf*
Tf		838	1436	1840	1077
Sc	4310		5038	6125	4007
Mt	2184	1764		2548	1413
Cd	1191	1058	1076		921
Nf	3196	2495	2894	4109	
Tf+Sc			673	1055	409
Tf+Mt		75		555	85
Tf+Cd		53	151		51
Tf+Nf		170	444	814	
Tf		2279	1681	1277	2040
Sc	3905		3177	2090	4208
Mt	1815	2235		1451	2586
Cd	1129	1262	1244		1399
Nf	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	**beta glucosidase/ cellobiase**		No		(46)
Tfu1629	GH_1	glucosidase/galactosidase		Yes	No	
Tfu0915	GH_2	galactosidase		No		
Tfu1607	GH_3	xylosidase/acetylhexosaminidase	β-1,3-glucan, CBM6	No		
Tfu2486	GH_3	xylosidase/acetylhexosaminidase		Yes		
Tfu2768	GH_4	glucuronidase/galactosidase/glucosidase		No		
Tfu0900	GH_5	**beta-mannanase** (EC 3.2.1.78)	Cellulose, CBM2	Yes	Yes, RKRLAVAAATVLALLASVFALTQPANAAT	(14)
Tfu0901	GH_5	**endocellulase (E5)**	Cellulose, CBM2	Yes	Yes, RKGGPPVAVAVTAALALLIALLSPGVAQAAG	(28)
Tfu2712	GH_5	**Cellulase**	Cellulose, CBM3	Yes	Yes, RRLRAGAAAIAIGASALIPLTSSPAAASG	(38)
Tfu0620	GH_6	**beta 1,4 exocellulase (E3)**	Cellulose, CBM2	Yes	Yes, RRSWMRRGLAAASG	(28, 53)
Tfu1074	GH_6	**beta 1,4 endoglucanase (E2)**	Cellulose, CBM2	Yes	No	(28)
Tfu1627	GH_9	**beta-1,4 endoglucanase (E1)**	Cellulose/amorphous/xylan, CBM2, CBM4	Yes	Yes, RRPRSRSPLVAL	(19)
Tfu2176	GH_9	**Cellulase (E4)**	Cellulose, CBM2, CBM3	Yes	Yes, PRRRGRHSRARRF	(19, 28)
Tfu2791	GH_10	**Xylanase**		Yes	No	(22)
Tfu2923	GH_10	**Xylanase**	Cellulose, CBM2	Yes	Yes, RHRPSRRARRSLSLLLTSALTAAGLLVTAAPAQAES	(4)
Tfu1213	GH_11	**endo 1,4 beta D xylanase**	Cellulose, CBM2	Yes	Yes, RRRFRPRLLIGKAFAAALVAVVTMIPSTAAHAAV	(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, RRRTFAPTWVVLLVAAGVVALC	
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	**beta 1,4 exocellulase (E6-celF)**	Cellulose, CBM2	Yes	Yes, RRWRTLASGALAAALAAAVLSPGVAHAAV	(18)
Tfu3044	GH_65	trehalase/maltose phosphorylase		No		
Tfu1612	GH_74	**Xyloglucanase, endo-β-1,4-glucanase**	Cellulose, CBM2	Yes	Yes, RRRGIIARALTCIAAAATVAAVG	(17)
Tfu2205	GH_77	amylomaltase or 4--glucanotransferase		No		
Tfu2130	GH_81	**Glucanase**		Yes	Yes, RRRWRRATTSAATAALLCGALLTFPSAPAAA	(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, VGRSITRRLASTLATAAVVTAGLTLPVSPASAQT	
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, RRRPVRFGAALAAFVLGATGAAALPSPAHAAA	

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		

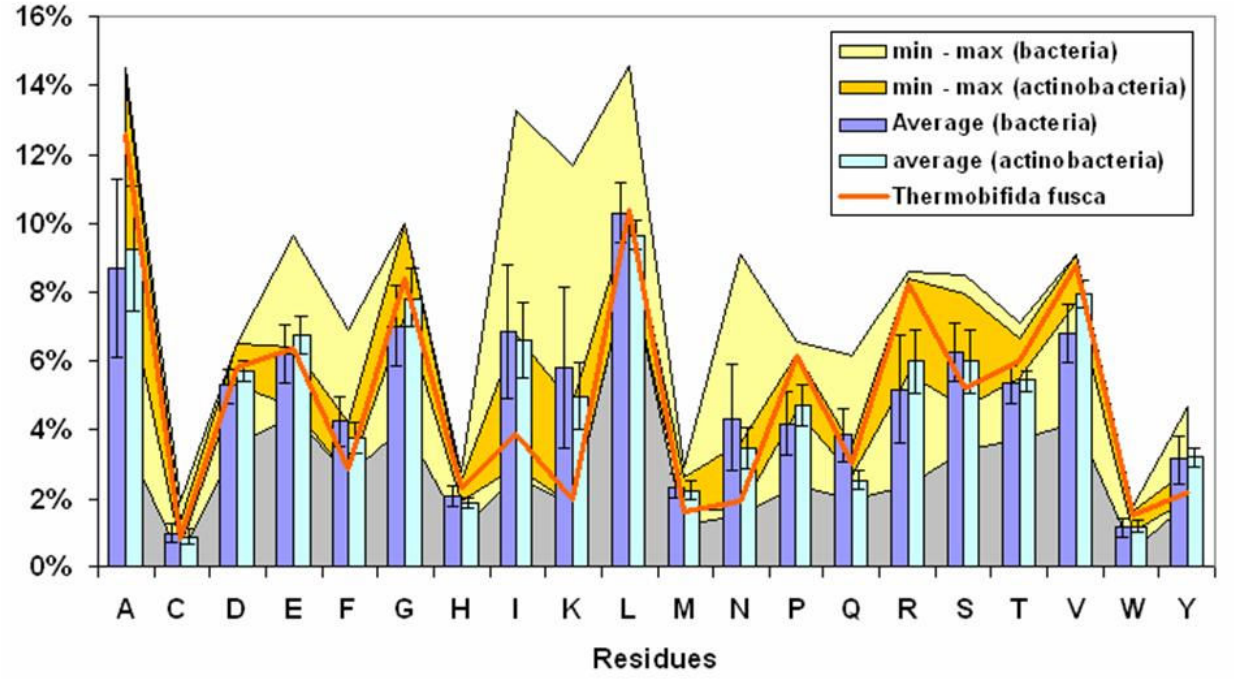

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Figure 2

Figure 3

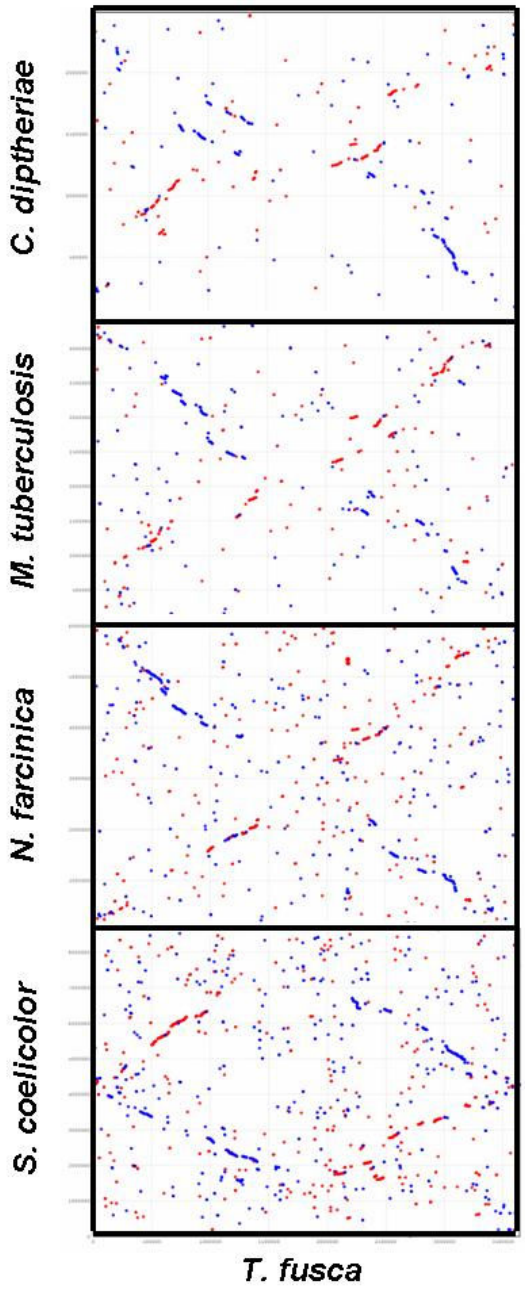
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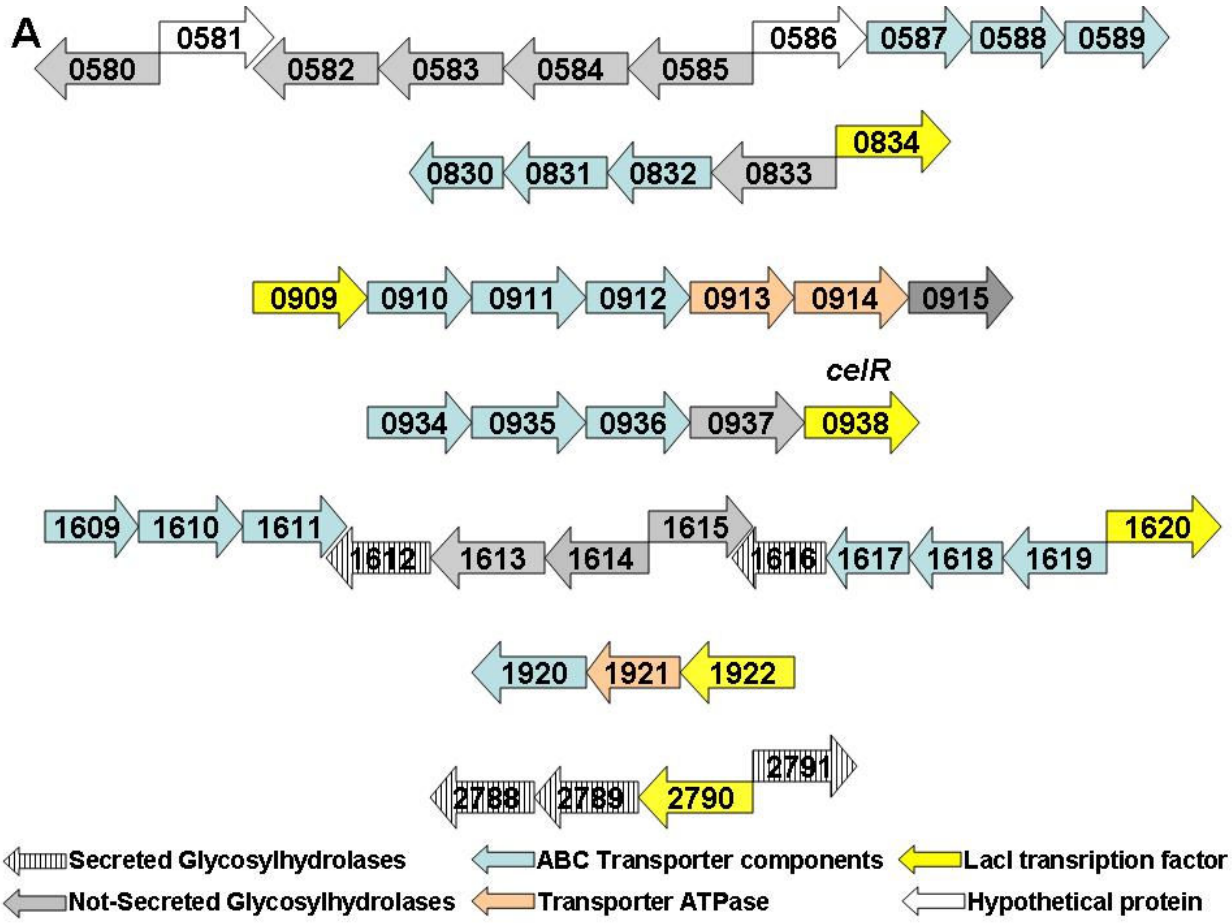
Figure 4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LacI transription factor
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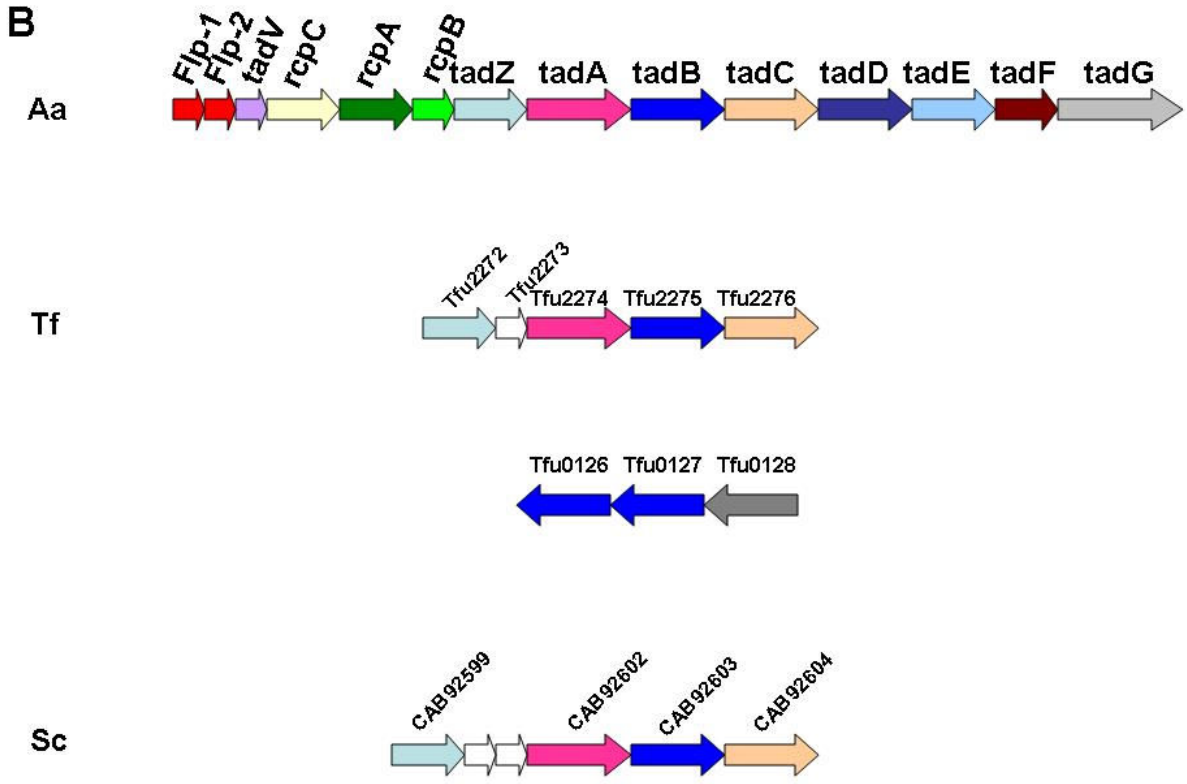
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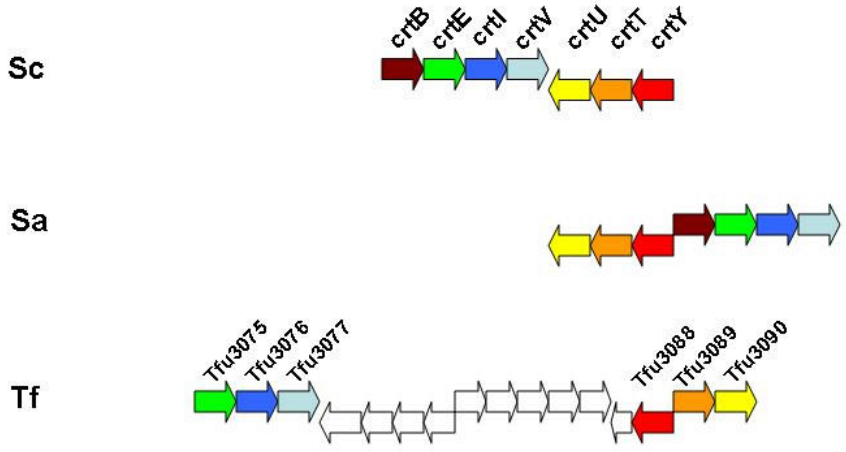
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