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Comparative genomics analyses on EPS biosynthesis genes required for floc formation of *Zoogloea resiniphila* and other activated sludge bacteria

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ABSTRACT

Activated sludge (AS) process has been widely utilized for municipal sewage and industrial wastewater treatment. *Zoolgoea* and its related floc-forming bacteria are required for formation of AS flocs which is the key to gravitational effluent-and-sludge separation and AS recycling. However, little is known about the genetics, biochemistry and physiology of *Zoogloea* and its related bacteria. This report deals with the comparative genomic analyses on two *Zoogloea resiniphila* draft genomes and the closely related proteobacterial species commonly found in AS. In particular, the metabolic processes involved in removal of organic matters, nitrogen and phosphorus were analyzed. Furthermore, it is revealed that a large gene cluster, encoding eight glycosyltransferases and other proteins involved in biosynthesis and export of extracellular polysaccharides (EPS), was required for floc formation. One of the two asparagine synthase paralogues, associated with this EPS biosynthesis gene cluster, was required for floc formation in *Zoogloea*. Similar EPS biosynthesis gene cluster(s) were identified in the genome of other AS proteobacteria including polyphosphate-accumulating *Candidatus* Accumulibacter phosphatis (CAP) and nitrifying *Nitrosopira* and *Nitrosomonas* bacteria, but the gene composition varies interspecifically and intraspecifically. Our results indicate that floc formation of desired AS bacteria, including CAP strains, facilitate their recruitment into AS and gradual enrichment via repeated AS settling and recycling processes.

1. Introduction

The activated sludge (AS) process has been widely applied for municipal sewage and industrial wastewater treatment for a century. However, AS process is facing great challenges such as sludge bulking, inefficient phosphorus and nitrogen removal and expensive surplus sludge disposal (Jenkins et al., 1993; Wanner, 1994). Due to the huge amount of sewage and wastewater treated by AS process worldwide, even a little improvement in the existing technology will have profound impact on the global economy and environment. Bacterial floc formation plays a central role in AS process. Butterfield et al. (1937) isolated a zoogloea-forming organism from activated sludge and tentatively identified it as *Zoogloea ramigera*. The bacterial culture was capable of forming flocs and exhibited oxidation characteristics similar to those of normal activated sludge (Butterfield et al., 1937). Therefore, he believed that *Z. ramigera* was the only bacterium of any consequence in AS. This bacterium had long been considered to be the typical microorganism responsible for the formation of activated sludge flocs. Relatively high levels of *Z. ramigera* cells and typical morphology

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tended to be linked to overloading sewage plants (Rosselló-Mora et al., 1995). The extracellular polysaccharides (EPS) secreted by AS bacteria including *Zoogloea* play a central role in the formation of matrix of multicellular conglomerates called zoogloeal flocs. Formation of zoogloeal flocs makes it possible to separate treated liquor from sludge by gravitational precipitation in the clarifier (settlement tank) and readily to recycle the settled AS back to the aeration tank for maintaining high cell densities in the mixed liquor to accelerate water purification process, two hallmark features key to the success of AS process. On the other hand, viscous (non-filamentous) bulking of activated sludge is characterized by the excessive growth of *Zoogloea*-like microorganisms and the production of excessive amounts of viscous extracellular polymeric substances, mostly polysaccharides, when nitrogen is deficient (Novák et al., 1994; Peng et al., 2003).

Microbiological researches have been increasingly conducted on activated sludge for optimization and renovation of AS process. During 1960s, several floc-forming bacterial strains were isolated from AS and identified as *Z. ramigera* (Dugan and Lundgren, 1960; Crabtree and McCoy, 1967; Unz and Dondero, 1967a). As suggested by Richard F. Unz, the *Z. ramigera* ATCC 19544 (=N.C. Dondero 106 strain), directly isolated by microdissection of a natural branched zoogloeal floc (Unz and Dondero, 1967b), had finally been adopted as the neotype for the species of *Z. ramigera* Itzigsohn (1868) (Unz, 1971; Skerman et al., 1980). The previously proposed neotype strain *Z. ramigera* ATCC 19623 (=K. Crabtree I-16-M) was reclassified in a new genus of *Rhizobiales* and renamed *Shinella zoogloeoides*, while another suggested neotype strain *Z. ramigera* ATCC 25925 (=P.R. Dugan 115 strain) was renamed as *Duganella zoogloeoides* of *Burkholderiales* based on 16S rRNA gene sequence analysis (Hiraishi et al., 1997). Three oligonucleotide probes complementary to the 16S rRNAs of *Z. ramigera* ATCC 19544 were designed to detect the bacterial cells that accumulated in the typical branched gelatinous matrices, the so-called *Zoogloea* fingers (Wagner et al., 1993; Rosselló-Mora et al., 1995) and it was shown that *Zoogloea* cells accounted for up to approximately 10% of the total cell numbers. The 16S rRNA gene clone library sequencing showed that *Proteobacteria* was the largest phylum in the activated sludge (Snaidr et al., 1997) and the PhyloChip microarray analyses further revealed that *Proteobacteria* was the predominant phylum, followed by *Firmicutes*, *Actinobacteria* and *Bacteroidetes* (Xia et al., 2010). As revealed by high throughput 454 pyrosequencing of amplicons, *Zoogloea* was one of the dominant genera and existed in high abundance (1.38–11.1%) in most of the AS samples from the municipal sewage treatment plants of mainland China, Hong Kong, Singapore, Canada, and United States (Zhang et al., 2012; Wang et al., 2012). However, *Zoogloea* was not the predominant species in the industrial wastewater-treating activated sludge (Ibarbalz et al., 2013; Ma et al., 2015).

The species diversity of the *Zoogloea* genus has been gradually revealed and so far three *Zoogloea* species, *Zoogloea ramigera*, *Zoogloea resiniphila*, and *Zoogloea caeni*, have been isolated from the activated sludge and identified taxonomically (Shin et al., 1993; Mohn et al., 1999; Shao et al., 2009). Two other *Zoogloea* species, *Zoogloea oleivorans* and the free-living diazotroph *Zoogloea oryzae* have been isolated from a petroleum hydrocarbon-contaminated site in Hungary (Farkas et al., 2015) and the soil from a rice paddy field in Japan (Xie and Yokota, 2006), respectively. We had isolated two strains of *Zoogloea* from the municipal sewage treatment plant located in the Wuhan City, Hubei Province and Hong Kong, respectively, and both strains were tentatively identified as *Z. resiniphila* based on 16S rRNA sequence using species cutoff of 97% identity. Recently we conducted the draft genome sequencing and annotation to reveal the metabolic traits and floc-formation relevant to the water purification performance of this important AS bacterium. We have identified a large gene cluster, including two asparagine synthase paralogues, required for the floc formation of *Zoogloea*. We also conducted comparative genomics analyses among the closely related AS proteobacteria *Zoogloea, Azoarcus, Thauera*, *Dechloromonas, Candidatus* Accumulibacter and *Nitrosospira* strains. Our results indicate that those AS bacteria, including the yet-uncultured polyphosphate-accumulating *Candidatus* Accumulibacter phosphatis (CAP) strains (Hesselmann et al., 1999; Albertsen et al., 2012; Flowers et al., 2013), harbor the EPS biosynthesis gene cluster(s) required for formation of flocs, thereby facilitating their enrichment in the AS process.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Supplementary Table S1. Bacterial strains were cultured in Luria-Bertani broth (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, pH 7.0)/agar plates, R2A agar plate (Reasoner and Geldreich, 1985), a *Zoogloea* medium (ZM) containing 0.5% casamino acids, 0.5% yeast extract, 0.2% K_2HPO_4 and 0.1% KH_2PO_4 (Fukui et al., 1976) or the modified M1 minimum media (supplemented with 15 μg/ml of gentamycin or 50 μg/ml of kanamycin when necessary). The *Zoogloea* strains were incubated at 28 °C in our laboratory.

2.2. Genome sequencing, annotation and bioinformatics analyses

The DNA sequencing, assembly, and annotation of the *Zoogloea resiniphila* genomes were conducted by using the Illumina Hiseq 2000 platform, the SOAP *de novo* version 2.21 package and RAST (Rapid Annotation using Subsystems Technology) server, respectively. The Clustal Omega package was used for polypeptide sequence alignments. The phylogenetic tree was constructed by neighbor joining method with CLUSTAL X and MEGA 6.0 programs.

2.3. Carbon source and electron acceptor utilization assays

BioLog phenotypic assays (Microplate GenIII) and the minimum media (supplemented with different carbon or energy sources) were used to monitor the utilization of carbon sources by *Zoogloea resiniphila* strains.

2.4. Transposon mutagenesis, in-frame deletion and genetic complementation

The *mariner* transposon mutant library (pMini*Hmar* RB1, courtesy by Dr. Daad Saffarini) was prepared (Bouhenni et al., 2005). Transposon insertional mutants (Km^r) were screened and isolated on R2A agar plates (1.5% agar, w/v) supplemented with 50 μg/ml of kanamycin. The transposon insertion sites were mapped as previously described (Bouhenni et al., 2005). For in-frame deletion mutants, the two-step protocol of selection (single cross-over, antibiotic resistance) and counter-selection (double crossover, sucrose sensitivity) was conducted using the suicide vector pDS3.0 (R6K replicon, *sacB*, Gm^R)-based constructs with a fusion of upstream and downstream sequences of the target gene as previously described (Wan et al., 2004). For genetic complementation analyses, the target genes were PCR amplified and cloned into the pBBR1-MCS5 or pHERD30 vector (Qiu et al., 2008). The resultant constructs and empty vector were transferred into the *Zoogloea* wild type strain or mutant strains via conjugation using *Escherichia coli* WM3064 as a donor strain.