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Darren Dunlap

Characterization of Phaeobacter sp. Y4I random transposon mutants with altered motility phenotypes

Darren Dunlap

May 2008

Introduction

Open water and coastal marine ecosystems offer a wide abundance of microbial flora, one of the most abundant of which resides in members of the Roseobacter lineage in the α-proteobacteria (2). Many Roseobacter have been isolated and identified in southeastern U.S. estuaries, where they can be found in large numbers (4). The ability of isolated representations to break down lignin and lignin-related monomers indicates that the lineage may play an important role in the breakdown of aromatic compounds in nature (1). Because the clade is easily cultured in the laboratory, Roseobacters makes ideal candidates for ecological study to determine their role in the natural habitat (3). One major aspect in the understanding of this clade lies in the use of genetic approaches to determine the components of its genome.

Genomes of many Roseobacter strains have been sequenced and completed in recent years, allowing for closer study of the relationship between strains and more efficient methods of genotypic analysis. While a total of 23 Roseobacter strains have completed genomes and 10 strains are soon to follow, many of these strains have yet to be properly characterized (8).

While there are many isolates of the Roseobacter lineage, the focus of this study is on *Phaeobacter* sp. strain Y4I, which harbors many interesting characteristics. Under certain conditions, Y4I produces the insoluble, blue pigment indigoidine, forms biofilms, and, most importantly, has a high rate of motility. The genome of Y4I was studied using aTn5 based transposon mutagenesis strategy (5). A modified version of the Tn5 transposon that was smaller in size and contained an antibiotic resistance marker is inserted into a carrier plasmid. The plasmid is introduced into Y4I through conjugation with *E. coli.* Once the plasmid is transferred, the transposon inserts itself into a single position in the Y4I genome, interrupting transcription at the point of insertion. Optimized conditions were used to generate a mutant library of ca. 6000 mutants that was then screened for various phenotypes, including altered motility, pigmentation, and production of antimicrobial compounds.

This study focuses on the Y4I transposon mutants exhibiting altered motility phenotypes. Out of 65 mutants studied, 10 exhibited hypermotile traits, 12 were slower than wildtype, and three had greatly reduced motilities. In addition, the site of transposon insertion was determined for each of these strains in order to connect the mutated trait with the underlying gene malfunction. The shutoff of transcriptional

regulators and polar amino acid transport genes were connected with hypermotile behavior, while the shutoff of ATP-dependent C1p protease, sulfate adenylytransferase, and histidine sensor kinase genes were connected with causing slower motility than wildtype Y4I.

Methods

Determining Kanamycin Concentration

Wildtype Roseobacter strain Y4I with and without the maintainable plasmid harboring the kanamycin resistance gene (pRK415ΩKmR) was tested against varying concentrations of kanamycin (Km) in broth and on solid media. Broth assays were performed in test tubes with 10 mL YTSS media, containing 2.5 g yeast extract, 4 g tryptone, and 15 g sea salts (Sigma-Aldrich) per liter. YTSS plates contained 15 g granulated agar. Aliquots of a Km 10 mg/ml stock was added to both broth and solid media to the final concentrations of 0, 5, 10, 15, 25, and 50 mg/L. Both wildtype and Y4I strains harboring pRK415ΩKmR were grown on YTSS plates overnight at 30°C. Individual colonies were inoculated into YTSS broth with varying concentrations of the antibiotic and placed in a 30°C shaking incubator overnight. Overnight liquid cultures were streaked onto YTSS plates containing varying concentrations of Km and incubated overnight at 30°C. ***50 mg/L was found to be the optimum concentration of kanamycin for selective growth for the strain expressing kanR.***

Creating a Mutant Library using Random Transposon Mutagenesis

To randomly insert the Tn5 transposon containing the Kanamycin-resistance gene into the Y4I genome, *E. coli* strain EA145, which harbored a plasmid with the Tn5 transposon, was mated with Y4I. Overnight cultures of Y4I and EA145 grown in YTSS, were inoculated together in YTSS broth overnight at 30°C. A 1:1 Y4I:EA145 ratio was used for the mating, with 200 μL of each strain inoculated into 10 mL YTSS broth. EA145 and Y4I were also incubated alone as controls. After the mating period, a 1:10 550 μL dilution of the mating mixture was plated onto large 50 mg/L Km YTSS plates (~5.5x the size of average-sized Petri plates—measure the sides and provide as # x # cm square Petri dishes) and incubated at 30°C for three days. Each large plate would contain 1000 – 1,200 mutant colonies.

To create the mutant library 6,144 colonies of varying size and pigmentation were selected and patched onto large 50 mg/L Km YTSS plates with sterile toothpicks in four by 98 grids. A 98 pin replicator facilitated transfer of mutants from the master plates to screening media.

Screening the Y4I mutant library for hypermotile mutants

To select for potential hypermotile mutants, preliminary motility assays were run in one-tenth YTSS semi-solid (0.35%) agar (motility plates), consisting of 0.25 g yeast extract, 0.4 g tryptone, 15 g sea salt (Sigma), and 3.5 g purified agar (Sigma). The mutants were grown in broth YTSS, shaking at 30°C overnight. The motility plates were inoculated with the mutants and were placed upright in a plastic container lined with moist paper towels to prevent dehydration of agar plates. The container was then sealed and placed in a 30°C incubator overnight. Y4I hypermotile mutants exhibited a wider diameter or growth over time, as compared to wild type Y4I.

Motility Assay

All motility assays were run on motility plates (described above). Wildtype and mutant Y4I were grown in broth YTSS, shaking at 30°C overnight, for at least two transfers to deter floccing of cells. Motility plates were inoculated with strains of interest in the center of a plate. This was done in triplicate. Plates were placed upright in a plastic container lined with moist paper towels to prevent dehydration of agar plates. The container was then sealed and placed in a 30°C incubator. Distance migrated from the center point was measured every 24 hours for three days. After 72 hours, fresh motility plates were inoculated by taking 15 µL from the leading edge of growth in the previous plate and inoculating the center of a fresh plate. This process was repeated three times for each strain of interest.

Genotypic Analysis using Arbitrarily-primed PCR (AP-PCR)

Genomic DNA was isolated from bacterial strains using the Qiagen Tissue Extraction Kit protocol (Qiagen, Valencia, CA). DNA was eluted from columns with 30 µl of diH2O and stored at -20°C.

In order to determine the insertion site of the transposon an arbitrarily-primed polymerase chain reaction (AP-PCR) approach was used (7). The AP-PCR process consists of two consecutive reactions

which amplify the DNA flanking the inserted transposon; in the first reaction, the forward primer is complementary to the transposon and the reverse primer contains a random sequence on the 3' end and a nucleotide tag with a known sequence on the 5' end. In the second PCR reaction, the forward primer is complementary to a region of the transposon internal to that amplified in the first round of amplification. The reverse primer is complementary to the tag incorporated into the amplicon in the first round of PCR (Figures 1.0, Table 1.0).

AP-PCR Reaction 1 was run in 50 µl reactions containing 5 µL 10x GoTaq Reaction Buffer (Promega, location), 1 µL dNTP mix (Promega, Wisconsin), 1 µL arbitrary primer (ARB1), 1 µL Out primer, 0.25 µL GoTaq Taq polymerase (Promega), 41.75 µL MilliQ water, and 100 ng of genomic DNA. AP-PCR Reaction 2 was run in 100 µL reactions containing 10 µL 10X GoTaq Reaction Buffer, 2 µL dNTP mix, 2 µL arbitrary primer 2 (ARB2), 2 µL Nest primer, 0.5 µL GoTaq Taq polymerase, 5 µL from reaction 1, and 78.5 µL diH_2O. Refer to Table 1.1 for AP-PCR thermocycle conditions.

After amplification, the PCR products were cleaned using the Qiagen PCR Purification Kit and the purified products submitted to the University of Tennessee's Molecular Biology Sequencing Facility. DNA sequences were subjected to homology searches (BLASTX) against the National Center of Bioinformatics (NCBI) database.

Results

Determining Kanamycin Concentration

Both wildtype and kanamycin-resistant strains were empirically tested for growth on liquid and solid YTSS media containing varying concentrations of kanamycin. Growth data can be found in Tables 1.2 and 1.3. As stated in the methods section, 50 mg/L was selected as the optimal concentration in broth and solid media for positive selection of Y4I mutants harboring the Tn-5 transposon.

Screening the Mutant Library

After observing the mutant library outward growth on motility media, ten mutants were initially selected for potential hypermotility (Figure 1.1). These mutants were later tested with further motility assays to verify this designation.

Motility Assay

The migration speeds of the mutants in the motility assay were compared to the speed of the parent, wildtype Y4I. The percent difference between mutant and wild type was calculated to make these comparisons. If the percent difference of the migration speed of the mutants was greater than or equal to 15%, then the mutant was designated as hypermotile. Mutants exhibiting migration speeds of -15% or greater were designated as "slow", and speeds greater than -50% were designated as "negative." Five of the original ten strains of interest were designated as hypermotile. These mutants deviated from wildtype by as little as 15% to as much as 31%. As the motility assays were continued on additional Y4I mutants, more hypermotile mutants were found and added. Out of 65 mutants that were assayed, 10 mutants were designated as hypermotile. The corresponding data can be found on Table 1.4.

Genotypic Analysis

The results from the homology searches (BLASTX) against the NCBI database can be found on Table 1.4. Even though only 65 of the 6000+ mutants were subjected to genetic analysis, mutants still had transposon insertions in the same genetic locations. Two mutants that showed hypermotility both had insertions that showed homology to a polar amino acid uptake family ABC transporter gene. All mutants that had insertions at ATP-dep C1p and sulfate adenylyltransferase/adenylylsulfate kinase showed slow motility phenotypes.

There are a few individual mutant results that should be mentioned here. A mutant with the histidine sensor kinase gene (Y411AA7) interrupted showed a complete lack of motility, and did not migrate from the site of inoculation on the motility plate. Conversely, one mutant had the flagellar biosynthesis gene *flh*A interrupted, but still exhibited wild type motility.

Discussion

The purpose of this experiment is to identify and characterize potential genes in the genome of *Phaeobacter* species Y4I that are linked with its phenotypic motility. While the genome of Y4I has been sequenced (unpub. data, A. Buchan), a complete understanding of the genetic content of this organisms is not fully understood. There are two steps in the identification of genes – first, the site of the insertion is compared with any phenotypic traits that have been altered from wild type. This links a specific site within the genome to certain phenotypic traits. Second, the nucleotide sequence around the site of insertion is compared with known genetic sequences in other organisms. Similar genes can often be found in organisms of similar species, and finding genetic similarities between species can also help to characterize genes in the Y4I genome.

Ten hypermotile Y4I transposon mutants show significant increases in motility, which shows that some type of change has occurred in the mutant genome that resulted in the phenotypic difference (Table 1.4). Initial microscopy studies of each of the mutant strains did not show any significant differences in cell morphology, structure, or movement, indicating that the change most likely occurred in the processes inside the cell.

The BLASTX results help shed some light on the genetic changes that took place in the mutants. Three of the ten hypermotile mutants showed the potential site of the transposon insertion to be LuxR family genes. However, a look at the number of mutants containing inserts in LuxR family genes shows significantly more mutants with wild type motility than with enhanced motility. While there could be multiple LuxR genes with varying functions in the cell, there is not enough evidence from the data to make any official determination. The interrupted gene in two of the mutants was placed in indigoidine production genes. But because nine other mutants with hits in indigoidine production genes show wild type motility, as compared with two hypermotile mutants and one slow mutant, this finding is most likely an anomaly. Mutant Y413BC3, which shows hypermotile activity, has its transposon insertion site at transposase orfB. However, mutant Y408BH1 also has its insertion site at transposase orfB, and shows wild type motility. From the current data, no definite conclusions can be made about the effect of transposase orfB on motility.

The most significant results for hypermotility lie in the two mutants with insertions in a polar amino acid uptake family ABC transporter (PAAT), permease protein. These are the only two mutants with insertions at this gene, and both show hypermotile behavior. In this case, it would be wise to understand the lineage of the gene in question and its function in the cell. The NCBI homology search compared the Y4I sequence with the permease protein found in *Silicibacter pomeroyi.* This bacterium is found in the alphaproteobacteria, the same class as Roseobacter, which strengthens the possibility that a homolog is found in the Y4I genome. This gene is responsible for producing the membrane-bound permease protein that functions along with the ABC transporter. This particular permease allows for the selective uptake of polar amino acids into the membrane to be used by the cell, which is all driven by ATP (9). If interrupted by the transposon mutation, the cell would lose a lot of its capability to intake polar, hydrophilic amino acids, which is used in protein production in the cell. Because this mutation is associated with the production of proteins, it has complicated implications inside the cell, making it hard to pinpoint the exact mechanism that is causing the mutant to by hypermotile. If additional hypermotile mutants were discovered with the PAAT permease protein gene interrupted, it would give stronger evidence connecting this gene's inactivity with enhanced motility.

The next step would be to investigate the mutants with slow or greatly negative motility in regards to wild type. Mutant Y411AA7 was found to be completely non-motile, and its interrupted gene was in a histidine sensor kinase. Histidine kinases are used by the cell to monitor the environment, and regulate cell functions such as gene expression and chemotaxis (6). The loss of these environmental sensors for the cell would turn off the cell's ability to direct itself towards nutrient and chemical gradients, essentially turning the cell's motility off. The fact that the mutant completely lost its motility, combined with the nucleotide sequence's homology to the histidine kinase gene in other Roseobacter species, strongly supports the presence of the sensory box histidine kinase/response regulator gene in the Y4I genome.

The next mutant of interest has its interrupted gene is ATP-dependent C1p protease, which was found in 3 of the 65 mutants. ATP-dependent C1p protease is involved in protein metabolic processes, including ATP-binding, peptidase activity, and protein binding and folding (NCBI). While it is near impossible to pinpoint the exact mechanism that is inhibiting cell motility, it is possible to see how a

breakdown of protein metabolism could create a wide variety of phenotypic effects for the cell, potentially including cell motility.

The examination and characterization of the Y4I genome and its relationship with motility still requires a great deal of further research. First, more mutants in the mutant library need to be characterized. Examining more mutants will reveal additional genes for study and verification in the genome. Additionally, data regarding the effect of the mutations will be further supported as mutants with the same mutations, and thereby the same phenotypes, are discovered. At the time of this experiment, only 65 of a mutant library of 6000+ have been characterized, so there is still plenty of work to be done.

The next step in this study is to verify the exact point of transposon insertion in the genome. The point of interest lies in the fact that many mutants have the same site of transposon insertion. Naturally, it would seem that these mutants would have similar properties to other mutants with the same gene interruption. However, this is not always the case. One of the most significant examples involves mutants with the indigoidine gene *igiD* interrupted. Nine mutants of the 65 showed this insertion and the motility of the mutants range from -13% to +31% from that of wild type. Further analysis would help to determine the exact site of insertion to make sure the insertion is not flanking the gene of interest.

Finally, now that the genome of Y4I has been sequenced, the genes found from the homology searches can be aligned to the nucleotide sequence of the actual genome. Comparisons of the exact nucleotide sequences can reveal if the gene is truly present in the genome, and if the gene has mutated in any way in the species. More importantly, it is possible to begin to piece together and characterize the entire genome of Y4I, and find the sequence of genes present. This is the ultimate goal of this experiment, which can be pieced together as the mutants are studied and characterized further.

References

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Appendix: Tables and Figures

Figure 1.0 – AP-PCR Amplification

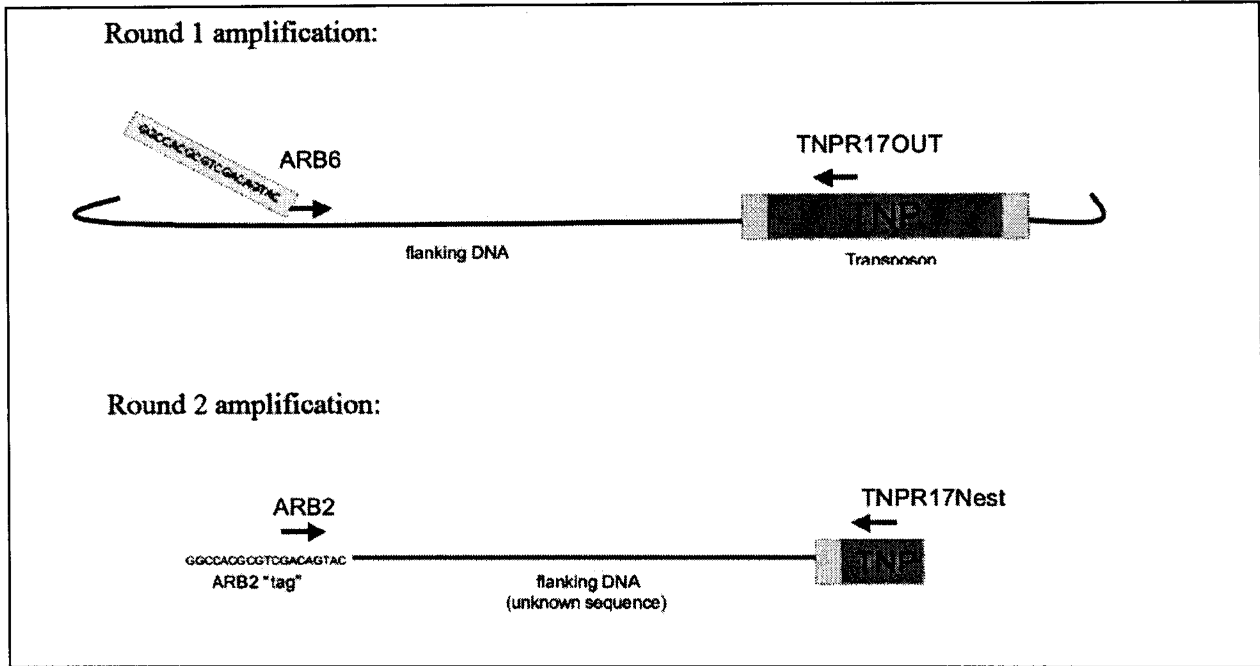

Table 1.0: AP-PCR Primer Pairing

1st Round Primer Pairs	2nd Round Primer Pairs	Sequencing Primer
ARB1 & TNRP17 OUT	ARB2 & TNRP17 NEST	TNRP17 NEST
ARB1 & TNRP13 OUT	ARB2 & TNRP13 NEST	TNRP13 NEST

Table 1.1: AP-PCR Round 1

	Round 1				Round 2			
Step	Temp (˚C)	Time (sec.)	Return to Step	Repeat	Temp (˚C)	Time (sec.)	Return to Step	Repeat
1	95	300	-	-	95	300	-	-
2	94	30	-	-	94	30	-	-
3	30	30	-	-	45	30	-	-
4	72	6	2	5x	72	6	2	30x
5	94	30	-	-	72	300	-	-
6	45	30	-	-	2	∞	-	-
7	72	60	5	30x				
8	72	300	-	-				
9	2	∞	-	-				

Table 1.2: Growth on Liquid YTSS Media containing Kanamycin

Strain	0	5	10	25	50
Y4I	+	+	+	-	-
Y4I (pRK415XXKmR)	+	+	+	+	+

Table 1.3: Growth on YTSS Solid Media plates containing Kanamycin

Strain	0	5	10	25	50
Y4I	+	+	-	-	-
Y4I (pRK415XXKmR)	+	+	+	+	+

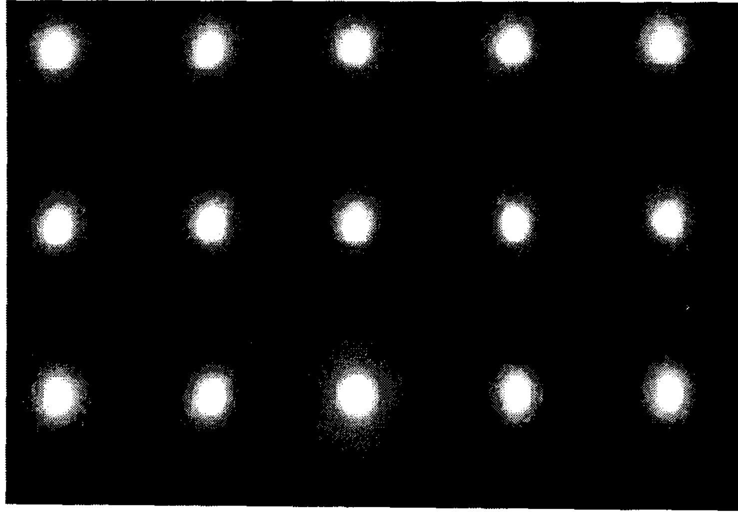

Figure 1.1: Initial selection for hypermotility

Table 1.4: Y4I Mutant Summary

Strain	Motility	% Dev	BLASTX Analysis (Closest Hit)	E-Value
Y4I	WT	-	-	-
Y402DA6	enhanced	32	Heat shock protein DnaJ-like, LuxR Family Autoinducer-binding transcript	5.00E-14
Y407BH10	enhanced	31	Indigoidine Sythesis	3.00E-03
Y412AH12	enhanced	31	response regulator (2comp Reg System)	3.00E-49
Y411BF5	enhanced	31	igiD	1.00E-64
Y402AE5	enhanced	27	LuxR, AHL(-)	7.00E-23
Y413BC3	enhanced	26	transposase orfB, possible LuxR family	2.00E-15
Y413BC1	enhanced	25	polar amino acid uptake family ABC transporter, permease	1.00E-17
Y408CC5	enhanced	22	hypothetical protein	0.19
Y405DD10	enhanced	19	igiC	3.00E-19
Y409DH10	enhanced	15	polar amino acid uptake family ABC transporter, permease	3.00E-3
Y414BE12	WT	14	methylenetetrahydrofolate reductase	5E-29
Y405BF3	WT	13	No Significant Hits	-
Y413CE4	WT	13	No Significant Hits	-
Y413BB4	WT	12	aminomethyl transferase family protein	1.00E-46
Y413BB7	WT	12	No Hits	-
Y413BC2	WT	12	Hypothetical Protein	3.00E-12
Y401CG4	WT	12	igiC	8E-32
Y413BB3	WT	11	aminomethyl transferase; glycyl-tRNA beta subunit	5.00E-37
Y413BB5	WT	8	No Significant Hits	-
Y411CD3	WT	7	IgiD	4.00E-34
Y402AH2	WT	6	possibly intergenic, upstream of transcriptional regulator	4.00E-43
Y401CC10	WT	6	Transcriptional Regulator, LuxR Family (E 3.7); low confidence	3.7
Y405CA6	WT	6	FlhA-, Flagellar Biosynthesis	2.00E-19
Y413BA11	WT	6	Hypothetical Protein	2.00E-13
Y401BA9	WT	6	unknown, possibly intergenic; upstream of oxidoreductase	1.00E-74
Y408AF7	WT	3	IgiD	6.00E-31
Y405CC11	WT	2	IgiD	1.00E-50
Y412CH6	WT	2	Hypothetical Protein	2.00E-13
Y412CH8	WT	2	actC domain protein (acetyltransferase)	3.00E-06
Y406CE12	WT	1	Lux R- transcriptional regulator	3.00E-41
Y412CH4	WT	1	unknown, possibly intergenic	9.00E-01

Y410AC12	WT	-1	LuxR, AHL(-)	1.00E-36
Y411CH9	WT	-1	unknown, possibly intergenic	1.4
Y409DE3	WT	-3	DNA-directed DNA polymerase	3.00E-67
Y412AE8	WT	-3	Hypothetical Protein	3.00E-09
Y411BG4	WT	-4	unknown, possibly intergenic; region upstream of LuxR-like trans. Reg.	1.00E-22
Y413BB2	WT	-4	probable phage protein	2.00E-05
Y406DC10	WT	-5	unknown, possibly intergenic	2.00E-30
Y406CE2	WT	-5	igiD	5.00E-22
Y405CD1	WT	-6	igiD	2.00E-59
Y411CE4	WT	-7	Lux R-type transcriptional regulator	2.00E-73
Y408BH1	WT	-7	Transposase orfB	4.00E-14
Y401BD4	WT	-8	igiD	9.00E-54
Y411CA11	WT	-8	conserved hypothetical protein	5.00E-17
Y406BD4	WT	-8	Transposase	5.00E-26
Y402AH10	WT	-9	unknown, possibly intergenic	3.00E-34
Y407AA10	WT	-10	unknown, possibly intergenic	2.00E-07
Y412AE6	WT	-12	LuxR, AHL(-)	9.00E-33
Y409AF8	WT	-12	igiB	2.00E-25
Y405DF10	WT	-13	Ppx/GppA phosphatase	4.00E-37
Y411BH1	WT	-13	IgiD	2.00E-30
Y416BH1	Slow	-16	ppGpp synthetase	2.00E-82
Y411A11	slow	-16	ATP-dep C1p protease	8.00E-45
Y416DD5	slow	-17	ATP-dep C1p protease	3.00E-31
Y410AD12	slow	-17	IgiB	1.00E-23
Y413CC6	slow	-18	ATP-dep C1p protease	1.00E-45
Y408BG6	slow	-20	Methyltransferase, putative	6E=22
Y406BH5	slow	-25	Sulfate adenylyltransferase/Adenylylsulfate kinase	5.00E-12
Y403AC3	slow	-31	Sulfate adenylyltransferase/Adenylylsulfate kinase	8.00E-40
Y401CG9	slow	-35	Hypothetical Protein	9.00E-05
Y413BB6	slow	-39	histidinol dehydrogenase	4.00E-41
Y415BB12	slow	-46	Hypothetical Protein	5.00E-19
Y415BB12	slow	-47	Hypothetical Protein	6.00E-19
Y406BG12	negative	-71	unknown, possibly intergenic	2.00E-35
Y403BE8	negative	-75	Phosphoadenosine phosphosulfate reductase	8.00E-40
Y411AA7	negative	-100	histidine sensor kinase	1.00E-07