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Genomic analysis of adaptive laboratory evolution of *Rhodococcus rhodochrous* strains – biocatalysts for acrylic monomers production

Shemyakina A.O.*, Grechishnikova E.G., Novikov A.D., Lavrov K.V., Yanenko A.S.
Kurchatov Genomic centre, National Research Centre "Kurchatov Institute", Moscow, Russia
** shemyakina.a.o@gmail.com*

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Motivation and Aim: A series of improved *Rhodococcus rhodochrous* biocatalyst strains for bioproduction of industrially significant acrylic monomers (acrylamide, acrylic acid, N-substituted acrylamides) was developed earlier in our laboratory using adaptive laboratory evolution (ALE) and further precise genome editing [1]. The ALE approach was chosen based on the hypothesis of co-regulation of nitrile hydratase (NHase) and amidase genes, conferring the corresponding activities, required for monomer production. The aim of this work was to track changes, appeared in the aforesaid genes, as well as in regulatory genes, which are assumed to control their expression. Additionally, we aimed to track whole-genome changes occurred during the ALE experiment, in order to identify the mutation types responsible for evolutionary plasticity of *R. rhodochrous* genome.
Methods and Algorithms: the genomes of *Rhodococcus rhodochrous* strains and their annotations [2] are available in the NCBI database under accession numbers NZ_CP064065.1, NZ_CP064066.1 (M8 strain genome and megaplasmid). NZ_CP129464.1, NZ_CP129465.1, NZ_CP129463.1 (M8-35 strain genome, 0.17 Mbp megaplasmid and 0.155 Mbp megaplasmid), NZ_CP129391.1, NZ_CP129392.1 (M8-20 strain genome and megaplasmid), NZ_CP129335.1, NZ_CP129336.1 (M8-33 strain genome and megaplasmid), NZ_CP129333.1, NZ_CP129334.1 (M8-50 strain genome and megaplasmid). Short changes (SNPs, indels) between the genomes were identified using Snippy [3] with default settings and --contig option. Larger changes (rearrangements) were identified using progressive Mauve algorithm [4]. Differences in amino acid sequences of enzymes associated with target activities were additionally verified using BLAST [5] algorithm supported by custom Python scripts.
Results: The wild-type *Rhodococcus rhodochrous* M8 strain which genome consists of a chromosome (6.1 Mbp) and a linear megaplasmid (0.17 Mbp) [6] served as the starting point of ALE experiment. Nitrile hydratase activity of M8 strain is determined by *nhmBA* genes, coding NHase β and α subunits [7]. Identification of genes responsible for amidase activity was complicated by the abundance of amidase genes (32) in M8 genome. The ALE experiment that aimed to exploit amidase phenotype of M8 strain consisted of 2 stages: (*i*) selection of clones with suppressed target activities and capable of growth in the presence of the toxic chloroacetamide (representative strain: M8-35) and (*ii*) obtaining revertant strains selected on medium that contained acetamide as the sole carbon source (representative strains: M8-20, M8-33, M8-50).
The results of tracking of changes in genes relative to target activities are summarized in Table 1. M8-35 was compared to wild-type genome and then served as a reference for

identifying mutations in genomes of revertant strains. Aside from enzyme genes mutations were concentrated in genes *nhmCD* of amide-dependent NHase transcription regulators. Their close homologues *nhlC and nhlD* are responsible for positive and negative regulation of corresponding NHase genes, respectively [8].

Table 1. Changes in genes relative to NHase and amidase activities in *R. rhodochrous* M strains series obtained in ALE experiment and their corresponding phenotypes

Gene name	Location	Changes occurred in				Product
		M8-35	M8-20	M8-33	M8-50	
amiE	Mega-plasmid	NC*	NC	Deletion	NC	Aliphatic amidase
fmdA1	Mega-plasmid	NC	NC	Deletion	NC	Acetamidase/formamidase
nhmC	Mega-plasmid	IS integration	NC	Deletion	Deletion	Amide-dependent NHase transcription regulator
nhmD	Mega-plasmid	NC	IS integration	Partial deletion	Deletion	Amide-dependent NHase transcription regulator
nhmB	Mega-plasmid	NC	NC	NC	Deletion	NHase subunit beta
nhmA	Mega-plasmid	NC	NC	NC	Deletion	NHase subunit alpha
amiN1	Chromosome	two FS**	NC	NC	NC	Amidohydrolase
amiN2	Chromosome	NC	NC	NC	FS	Amidohydrolase
Phenotype:	NHase	< 0.7	109.4	114.5	< 0.7	
	Amidase	< 1.5	106	12.4	210.4	

* NC – no changes. ** FS – frameshift mutation. Phenotype is expressed as percent of initial activity level of M8 strain.

Overall genome alignment revealed comparable scale of mutation background in all pairs of parent-child strains: about 200 point mutations, including SNPs (9–24 %), indels (74–90 %) and their combinations, and about 9 large (8 bp – 52 kbp long) rearrangements. Depending on the genomes being compared 24–62 % of point mutations led to changes in amino acid sequence of coding genes. The repertoire of large rearrangements included: IS relocations, tandem repeat (TR) contractions and expansions, gene duplication, 10s kbp long deletions and prophage integration; in half the cases the mutations were intragenic. Additionally, 48 % of large rearrangements that occurred during the second ALE stage were completely reversable, including recovery of ATP-binding protein gene (gen tag ISO16_RS07605) in all three revertant strains after IS mediated knockout in M8-35 strain. Besides IS relocations reversable cases belonged to TR copy number variation and the duplication of tRNA gene (Fig. 1). Aside from prophage integration another intercellular transposition was the appearance of second 0.155 Mbp long megaplasmid in M8-35 genome which however was not found in any of analyzed genomes of M8-35 offsprings.

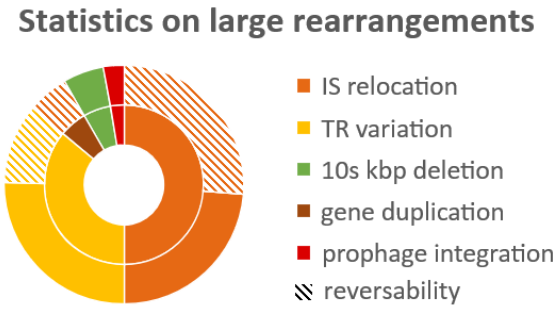

Fig. 1. Distribution of large rearrangements by type and reversibility in *R. rhodochrous* M strains series, obtained in ALE experiment

Conclusion: The two types of genome modifications that targeted the genes critical for NHase and amidase activities in M strains were gene disruption by IS integration and large deletions of unknown origin. In contrast, point mutations were much less associated with changes in key genes despite the considerable amount of indel induced frameshift mutations. Notably, most of key genes are located on the megaplasmid, which may indicate its evolutionary role in the metabolic adaptation of *Rhodococcus* bacterium to such recalcitrant substrates as nitriles.

In M8-33 and M8-50 strains the suppressed enzymatic activities conspicuously matched with deletions of corresponding enzymatic genes. Meanwhile the simultaneous switching of both activities in a row of M8 –> M8-35 –> M8-20 strains was accompanied by subsequent knockouts of the *nhmC* and *nhmD* genes. The fact that both knockouts were IS mediated leaves the possibility of full gene restoration as it was shown for another gene in this ALE set. Together these observations allow to assume the major role of *nhmCD* genes in nitrile-amide metabolism regulation in M8 strain.

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