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Genome-wide identification and characterization of genes encoding cyclohexylamine degradation in a novel cyclohexylamine-degrading bacterial strain of *Pseudomonas plecoglossicida* NyZ12

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ABSTRACT

The Gram-negative strain of *Pseudomonas plecoglossicida* NyZ12 isolated from soil has the ability to degrade cyclohexylamine (CHAM). The genes encoding CHAM degradation by gram-negative bacteria, however, have not been reported previously. In this study, ORFs predicted to encode CHAM degradation by NyZ12 were identified by bioinformatics analysis. Differential expression of the proposed ORFs was analyzed via RNA-seq and quantitative reverse transcription-PCR (qRT-PCR), using RNA extracted from NyZ12 cultured with or without CHAM addition. One CHAM-inducible ORF, *RK21_02867* predicted to encode a cyclohexanone monooxygenase (ChnB) was disrupted, as were five ORFs, RK21_00425, RK21_02631, RK21_04207, RK21_04637 and RK21_05539, that had weak homology to the only known cyclohexylamine oxidase (CHAO encoded by *chaA*) found in *Brevibacterium oxydans* IH-35A. We also found that a tandem array of five ORFs (RK21_02866-02870) shared homology with those in an operon responsible for oxidation of cyclohexanone to adipic acid, although the ORFs in strain NyZ12 were arranged in a different order with previously found in cyclohexane, cyclohexanol or cyclohexanone degradation strains. The ORFs in this cluster were all up-regulated when CHAM was supplied as the sole carbon source. When one of these five genes, *RK21_02867* encoding cyclohexanone (CHnone) monooxygenase, was knocked out, NyZ12 could not grow on CHAM, but it accumulated equimolar amounts of CHnone. Our results show that strain NyZ12 metabolized CHAM directly to CHnone which was then further metabolized to adipate. Despite clearly identifying genes encoding the steps for metabolism of CHAM metabolites, not every one of the putative *chaAs* was differentially expressed in the presence of CHAM and deletion of each one individually did not completely eliminate the capacity of NyZ12 to degrade CHAM, though it did reduce its growth in several instances. Our results suggest that there is genetic redundancy encoding the initial step in the oxidation of CHAM to CHnone in NyZ12 and that its CHAOs differ considerably from the ChaA, originally described in *Brevibacterium oxydans* IH-35A.

1. Introduction

Cyclohexylamine (CHAM) is an important chemical in industrial production of insecticides, plasticizers and antiseptics. It is also considered to be a hazardous pollutant, because of its toxicity via inhalation or skin contact, its potential carcinogenicity, and its refractory nature(Kroes et al., 1977; Petersen et al., 1972; Renwick and Williams, 1972). There are only two published reports of bacteria utilizing cyclohexylamine as a sole carbon and nitrogen source. The first was of metabolism by the Gram-positive bacterium *Brevibacterium oxydans* IH-35A isolated by Iwaki et al. (Iwaki et al., 1999b). The second was our own work in which we initially described CHAM metabolism by the Gram-negative bacteria *Pseudomonas plecoglossicida* NyZ12 (Shen et al., 2008). Iwaki et al. reported that CHAM degradation in strain IH-35A occurs via the following route: CHAM was first deaminated to cyclohexanone (CHnone) by the cyclohexylamine oxidase. CHnone was then oxidized to 6-hexanolactone, which was hydrolyzed to ring-cleaved product and further converted into adipate

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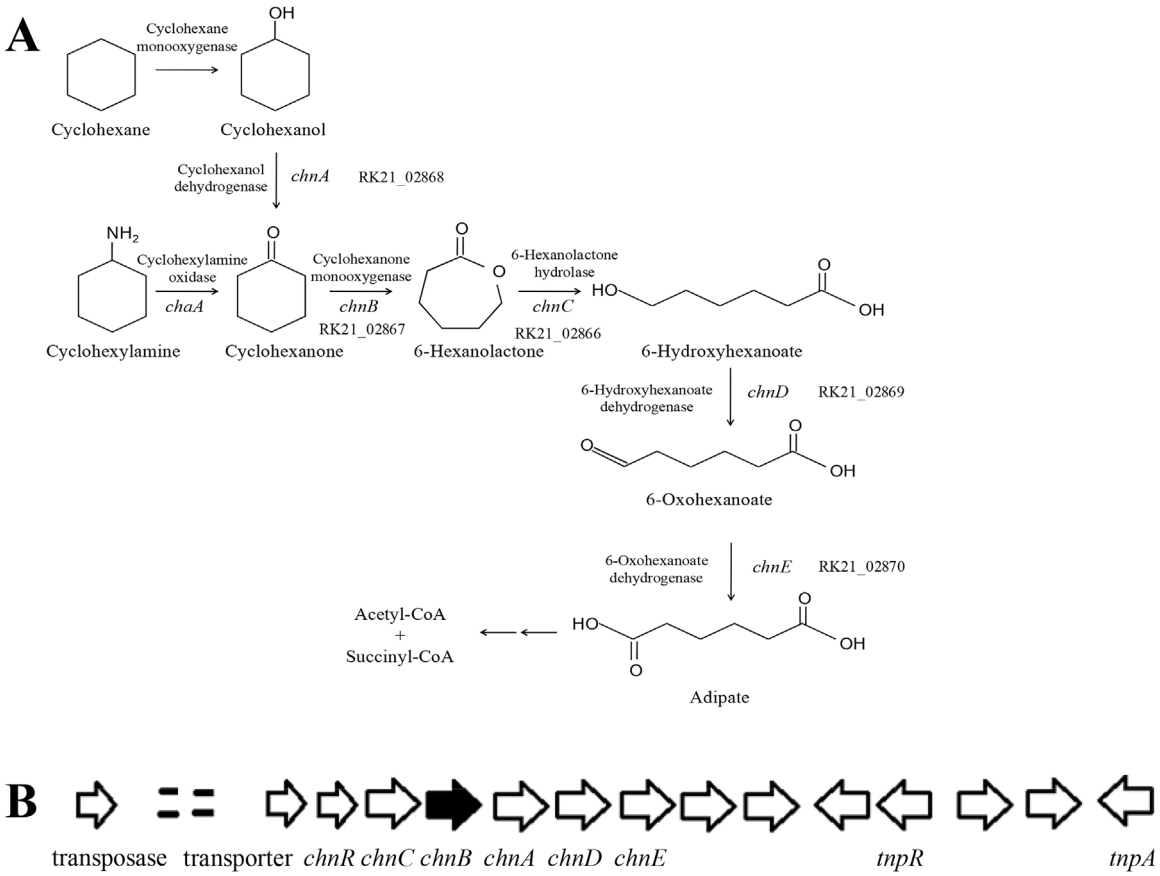

Fig. 1. (A) Proposed pathways for cyclohexlamine catabolism in *P. plecoglossicida* NyZ12 and *Brevibacterium oxydans* IH-35A. (B) Organization of the gene cluster responsible for cyclohexanol and cyclohexanone degradation in *P. plecoglossicida* NyZ12.

(shown in Fig. 1) as demonstrated by measurement of enzyme activities including cyclohexylamine oxidase (CHAO), cyclohexanone monooxygenase, 6-hexanolactone hydrolase, and 6-hydroxyhexanoate dehydrogenase (Iwaki et al., 1999b). Subsequently, the gene encoding cyclohexylamine oxidase, *chaA,* was cloned from *B. oxydans* IH-35A and shown to encode the degradation of cyclohexylamine to cyclohexanone when heterologously expressed (Mirza et al., 2013). Further characterization of ChaA including its physicochemical properties (Iwaki et al., 1999c), structure (Mirza et al., 2013), kinetic (Leisch et al., 2011), and substrate specificity (Li et al., 2014a) have also been reported.

CHnone was previously identified as an intermediate in the metabolism of cyclohexanol to adipic acid by *Acinetobacter* NCIB 9871 and *Pseudomonas* sp. (Donoghue and Trudgill, 1975; Tanaka et al., 1977). In bacteria capable of degrading cyclohexane, cyclohexanol, and CHnone, the genes encoding CHnone degradation to adipate are located on one gene cluster. Though the genes themselves share significant homology, their organizations in the clusters are genus-specific (Brzostowicz et al., 2005; Brzostowicz et al., 2003; Cheng et al., 2000; Iwaki et al., 1999a). To date, there have not been any reports characterizing the genes encoding CHnone oxidation in CHAM-degrading bacteria.

Recently, we sequenced and published the genome sequence of the cyclohexylamine-degrading *Pseudomonas plecoglossicida* NyZ12 (Li et al., 2015). In this study, homology-based inferences were used to identify ORFs potentially encoding the degradation of CHAM to adipate. Differential expression of those ORFs after induction with CHAM was investigated by RNA-seq and quantitative reverse transcription-PCR (qRT-PCR). To identify CHnone monooxygenease in NyZ12, gene deletion was made and one ORF, *RK21_02867*, was found to be the candidate gene as its knock-out mutant was unable to grow on CHAM and accumulated CHnone. In addition, The ORFs predicted to encode five monoamine oxidases (MAOs) were also knocked out individually in order to identify which gene(s) encoded CHAO activity in *Pseudomonas plecoglossicida* NyZ12.

2. Materials and methods

2.1. Strains, plasmids, and oligonucleotide primers

All strains, plasmids and oligonucleotide primers used in this study are listed in Table S1 in the supplemental material. *P. plecoglossicida* strains were generally cultivated in minimal salts medium (MSM) (Iwaki et al., 1999b) supplemented with 5 mM CHAM-HCl (MSM-CHAM) as the sole carbon and nitrogen source at 28 °C for 20 h with shaking at 150 rpm. Growth curves were determined by measurement of optical density at 600 nm (OD600).

For transcriptome analysis and qRT-PCR, strain NyZ12 was grown in MSM-CHAM or in MSM-succinate medium first, and then induced with 5 mM of CHAM, respectively, while strain NyZ12 grown in MSM-succinate was used as a control. When necessary, ampicillin, kanamycin, streptomycin and tetracycline were added at final concentrations of 100, 50, 10, 50 μg/mL, respectively.

2.2. Transcriptome analysis

Strain NyZ12 was grown in MSM-CHAM, while it was grown in MSM-succinate as a control. High quality RNA was isolated using a hot SDS/phenol method modified from Jahn et al. (Jahn et al., 2008). Total RNA was treated with RQ1 DNase (Promega) to remove genomic DNA. The quality and quantity of the purified RNA were determined by measuring the absorbance at 260 nm/280 nm using smartspec plus