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Genes Involved in the Biosynthesis of Lac Dye Constituents in Indian Lac Insect, *Kerria lacca* (Kerr)

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ABSTRACT

Kerria lacca is commercially harnessed for lac resin, dye and wax. This study is an attempt towards identifying the possible pathway and genes involved in the biosynthesis of the lac dye. It is proposed that lac insects employ polyketide pathway catalysed by polyketide synthase to produce laccaic acid D, a common precursor molecule for the biosynthesis of other lac dye constituents. Post-PKS tailoring enzymes are also involved in the alteration of polyketide chains. Thirteen related genes were identified and sequenced in this study.

Key words: Lac insect, laccaic acid, lac dye, polyketide, biosynthesis.

INTRODUCTION

Indian lac insects, *Kerria lacca*, belonging to the family Tachardiidae (=Kerriidae) which constitutes a specialized group in Superfamily Coccoidea (Hemiptera: Sternorrhyncha), show diverse body colours from crimson to yellow, and cream to albino (white). Colour difference in lac insect is inherited as a unit character and crimson is dominant to yellow (Sharma and Ramani, 2011). Wild type insect possesses crimson body colour due to the presence of a complex of closely resembling water-soluble polyhydroxy-anthraquinones (Fig. 1), collectively called as lac dye. Laccaic acids A and B are the principle components of lac insect body colouration (Bhide *et al*., 1969; Burwood *et al.,* 1967; Oka *et al*., 1998a, 1998b; Pandhare *et al*., 1966, 1967, 1969); whereas laccaic acid C, D and E have been isolated in little quantities (Mehandale *et al.,* 1968; Rama Rao *et al.,* 1968). The anthraquinone represented by laccaic acid D, is assumed as a parent compound for the biogenesis of all other anthraquinone pigment compounds found in wild type lac insects, such as laccaic acid A, B, C and E; and those found in related coccoids (for example, kermesic acid and carminic

acid from *Kermes ilicius* and *Dactylopius coccus*, respectively). Hu *et al.* (2011) isolated laccaic acid F from the Thai sticklac, *K. chinensis*. The amino acid residue in laccaic acid C appears to be derived from tyrosine by phenolic coupling with a polyhydroxyanthraquinone compound. Yellow mutant lac insects containing laccaic acid D have been reported (Chauhan, 1967; Negi *et al.,* 1945) in both *rangeeni* and *kusmi* infrasubspecific forms of *K. lacca, K. albizziae* and *K. fici*. The cream-coloured insect shows a very light yellow body colour (Ramani, 2011). White mutants lack both body and resin pigments and were observed by Chauhan and Teotia (1973) from F-4 progeny of a cross of two distant races of *K. lacca.* Both, Cream and white are recessive to the wild-type allele and are non-allelic to yellow.

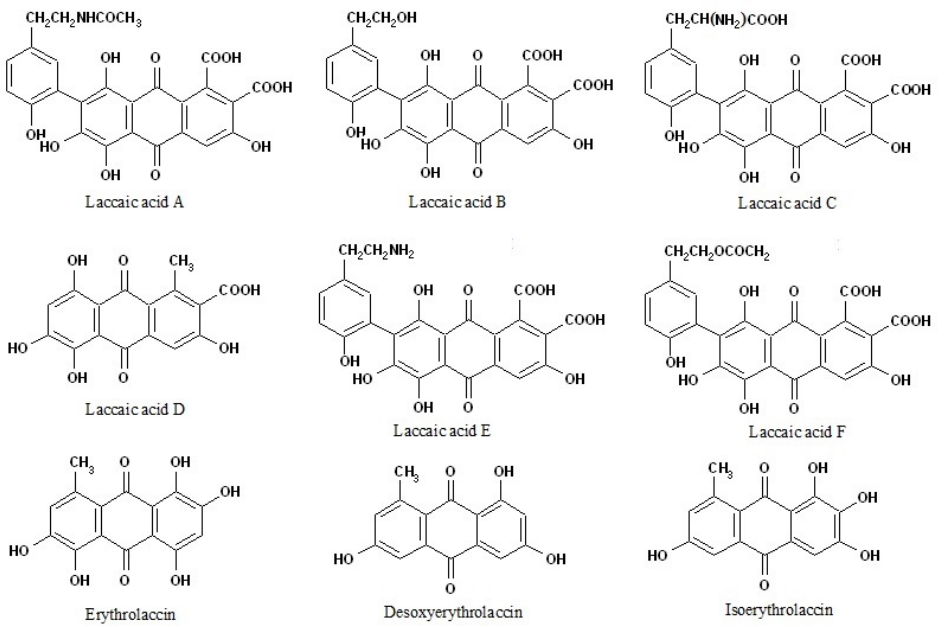

Fig. 1. Chemical structures of the components of lac pigment present in insect body as well as resin.

The protective resinous covering of the crimson and yellow lac insect also appears coloured; this yellowish hue is due to the presence of certain alcohol-soluble anthraquinone pigments related to laccaic acids, viz., desoxyerythrolaccin, erythrolaccin and isoerythrolaccin (Fig. 1). They also share a common biochemical pathway and are derived through a common precursor, laccaic acid D, which is a component of the body color pigments. Cream mutant insects show a very light resin colour whereas whites are devoid of any colouring pigments. Some studies on pigment production in other organisms have been reported. However, detailed studies on genes that are related to pigment production in *K. lacca* has not been reported so far. Therefore, with this first attempt, we aim to identify the biosynthetic pathway and genes responsible for anthraquinone biosynthesis in the Indian lac insect, *K. lacca* (Kerr).

Proposing biosynthetic Pathways leading to laccaic acids

The chemical nature of lac pigment studied suggests that laccaic acids ($C_{20}H_{14}O_{10}$) are hydroxy-anthraquinone caroboxylic acid derived through common precursor molecule laccaic acid D. The established literature reports two major biosynthetic pathways for anthraquinone biosynthesis; first is polyketide pathway and is characterized by the formation of anthraquinones by folding of a polyketide chain with both rings hydroxylated such as 1, 8-dihydroxylated anthraquinones (Han *et al*., 2001; Teuscher and Lindequist, 1994), and another is the shikimate pathway which is used to produce anthraquinones with only one hydroxylated ring like 1, 2 dihydroxylated anthraquinones.

Since, 1,8-dihydroxylated anthraquinones are biosynthesized by folding of a polyketide chain, therefore the structural similarities of these anthraquinones with laccaic acid D suggests that polyketide pathway, also known as acetate or malonate pathway is responsible for the synthesis of anthraquinones in lac insects (Fig. 2). Extensive literature review suggests that type II Polyketide Synthase (type II PKS) is the key enzyme for anthraquinoid pigment biosynthesis in lac insects. It was found in an experiment, that when *Streptomyces galilaeus* ATCC 31671, producers of 2-hydroxyaklavinone, were transformed with the DNA carrying the *actI, actVII, and actIV* loci (encodes type II PKS for actinorhodin biosynthesis) of *Streptomyces coelicolor*, the recombinant strain produced two novel anthraquinones, one is the 3-hydroxy analog of aloesaponarin II, desoxyerythrolaccin (the one which is a constituent of pigment found is lac resin and is derived from the parent molecule laccaic acid D), and 1-*O*-methyldesoxyerythrolaccin (Bartel *et al.*, 1990). Therefore, we suggest that lac insects also possess the type II PKS which has been extensively studied in *Streptomycetes* producing aromatic compounds, like e.g., actinorhodin.

In type II aromatic polyketide synthases, the functional domains are on discrete polypeptide chains (Hertweck *et al*., 2007), and they employ only the malonyl CoA extender units (Rawlings, 2001a, 2001b; Staunton and Weissman, 2001) resulting into the formation of whole polyketide chain followed by its cyclization, oxidation or reduction as required. A 'minimal type II PKS' consisting of two ketosynthase (KS) units, KS_α and KS_β/ CLF (chain length factor), and an acyl carrier protein (ACP), is usually sufficient to yield a polyketide chain of a defined length. In contrast to KS_α, the KS_β component is not capable of performing Claisen condensations due to the absence of cysteine residue. Apart from the decarboxylation activity, the KS_β is also the primary determinant of carbon chain length (McDaniel *et al*., 1995). Similar to the biosynthetic machinery operating in prokaryotes, the anthraquinone biosynthetic pathway in lac insects may also begin with the condensation of several acetate units. Upon catalysis by KS, decarboxylative condensation of malonyl CoA derivatives to acyl-thioesters (ACP or CoA) occurs to form β-ketoacyl thioesters (Fig. 3) (Austin and Noel, 2003; Smith *et al.*, 2003; White *et al*., 2005). In this reaction, which is common to both PKSs and fatty acid synthases (FASs), ACP carries a malonyl moiety at the thiol of a phosphopantetheinyl moiety attached to a serine residue. A fourth PKS component, malonyl/ acyltransferase (MAT/ AT) transfers the malonyl moiety of malonyl CoA to the thiol of the phosphopantetheinyl moiety attached to ACP. This phosphopantetheinyl

moiety is attached to ACP by the reaction of phosphopantetheinyl transferase (PPT) activity. In most type II PKS, a MAT/ AT is absent and malonylation activity is recruited from fatty acid biosynthesis (Bao *et al.*, 1998; Carreras and Khosla, 1998; Dreier *et al.*, 1999; Florova *et al.,* 2002; Khosla *et al.,* 1999; Revill *et al.*, 1995, 1996). Therefore, MAT provides a link between FAS and PKS in the same organism and shows the close relatedness between both synthases.

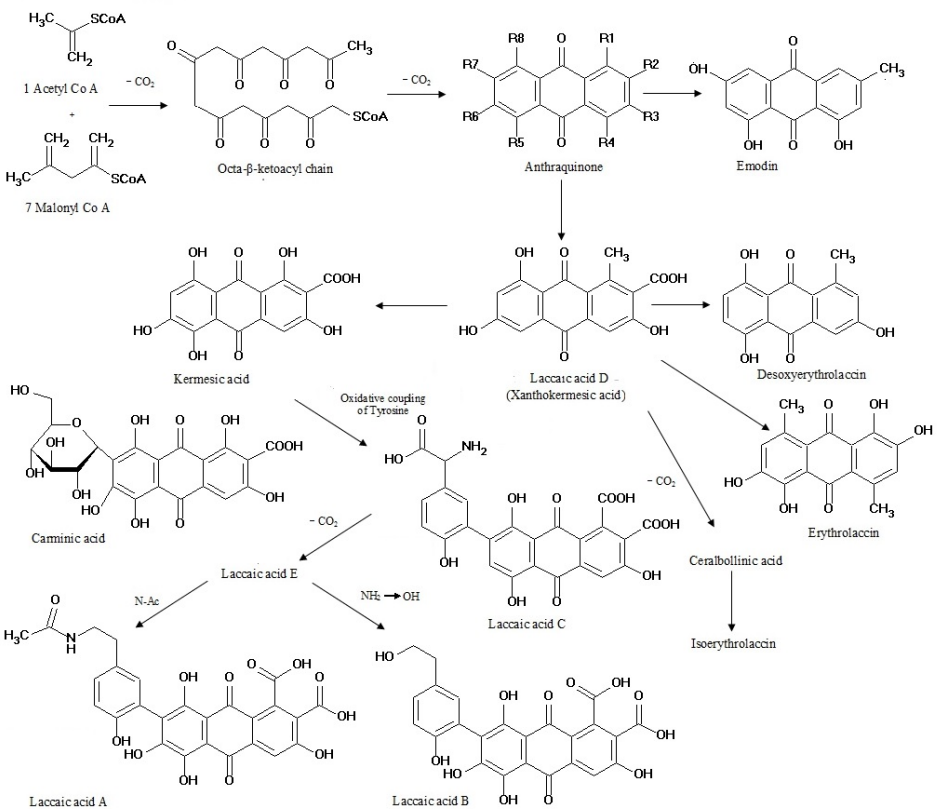

Fig. 2. Proposed pathway for the anthraquinone biosynthesis in scale insects focusing mainly on lac dye constituents (reproduced from Shamim *et al*., 2014).

The type II PKSs also contain various additional subunits, such as cyclases, aromatases and keto reductases, to process the polyketide chain attached to ACP into an initial frame. A controlled cyclization of the poly-β-keto chains into defined polyphenol structures is achieved only in the presence of cyclases and aromatases support the cyclodehydration process. The first ring cyclization largely depends on the minimal PKS and whether or not a ketoreduction has taken place (Fig. 3). These additional subunits are also called PKS-associated proteins, in contrast to post-PKS tailoring enzymes, which act after the release from the ACP. The most important and frequent post-PKS tailoring enzymes are oxidoreductases consisting oxygenases,

oxidases, peroxidases, reductases (e.g., ketoreductases), and dehydrogenases; glycosyl transferases; methyltransferases; halogenases and aminotransferases. These enzymes can introduce hydroxyl groups (hydroxylases), aldehyde or keto groups, and epoxides (epoxidases), or modify them by transforming a ketone into a secondary alcohol or an aldehyde into a carboxylic acid.

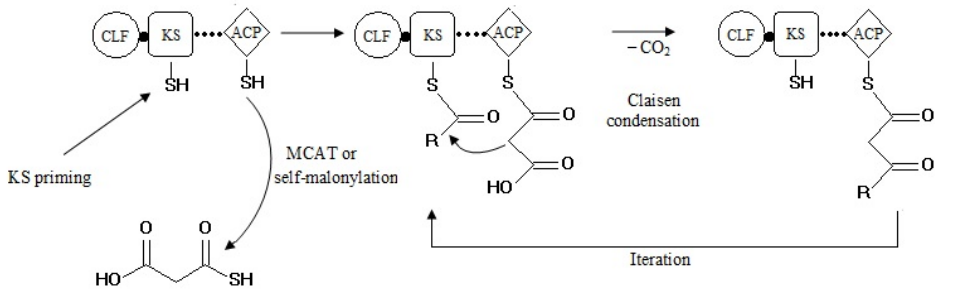

Fig. 3. Schematic representation of polyketide biosynthesis by minimal type II PKS; PKS represents the polyketide synthase, carrying two thiol groups, one on the ketosynthase (condensing enzyme; KS) and the other on the acyl carrier protein (ACP) (Rohr and Hertweck, 2010)

MATERIALS AND METHODS

Insect Materials and DNA Isolation

Indian lac insects, *K. lacca*, were derived from the culture maintained in the Field Gene Bank at National Lac Insect Germplasm Centre (NATLIGEC) of Indian Institute of Natural Resins and Gums, Ranchi, India (23°19'51"N 85°22'18"E; Elevation ~2080ft; Average temperature ~27ºC). The lac insect cultures were maintained on a common lac host *Flemingia macrophylla*, under potted conditions. The cultures were kept enclosed in synthetic mesh sleeves (60 mesh) to exclude the lac insects from parasite and predator infestations. The cultures were regularly sprayed with fungicide (carbendazim, 0.01%) to maintain the cultures clean. Mature female insects of four months (*rangeeni*) were collected and kept in 100% ethanol for 48 hours at room temperature to dissolve the resinous covering followed by cleaning the insects with sable hair brush under a stereo zoom microscope. Around 50-60 mg cleaned insects were weighed and kept at -80ºC overnight prior to the genomic DNA isolation. Genomic DNA was isolated using HipurA Insect DNA Purification Spin Kit (Himedia). For the present study, both wild type crimson and yellow mutant female insects were taken into consideration.

Primer designing, PCR Amplification and Sequencing

Primer designing to amplify the PKS sequences were based on the sequences of various ORFs of actinorhodin gene cluster in *Streptomyces coelicolor* A3(2) which encodes type II PKS and the PKS sequences of *Bombyx mori* and in the absence of information on genomic sequences of *K. lacca*; the specificity of the primers were

checked against Green pea Aphid, *Acyrthosiphon pisum* (Hemiptera: Aphidoidea), which is phylogenetically related to Indian lac insect. Primers were designed using online primer designing tool at NCBI, PRIMER-BLAST (Ye *et al.*, 2012).

The PCR reactions were performed in 50 µl reaction mixtures containing 40 ng of template DNA, 1X Taq buffer [100 mM Tris-HCl (pH 8.8), 500 mM KCl, 0.8% (v/v) Nonidet P40; Thermo Scientific, USA], 2.0 mM $MgCl_2$ (Thermo Scientific, USA), 0.2 mM of each dNTP mix (Affymetrix USB, Cleveland), 10 picomoles of each primers, 3 units of Taq DNA polymerase (Thermo Scientific, USA). The PCR reactions were carried out with the following cycling conditions: Initial denaturation of template DNA was carried out at 95ºC for 5 min followed by 35 cycles programmed for denaturation step at 95ºC for 30 sec, primer annealing step at specific temperature (see Table 1.) for 30 sec, and DNA extension step at 72ºC for 1 min. The final extension of the PCR products was carried out at 72ºC for 10 min. Some single primer amplification (Nainan *et al.*, 1996) was also performed.

Table 1. Detail of primers used for amplification of gene of interest in wild type and colour mutant lac insects

Primer ID	Sequences (5'- 3')	Tm (ºC)	Gene amplified	Amplicon length (bp)		Accession No.
				Crimson	Yellow	
4669_IM R	TGC AGA ATA CCA CGT AAA CA	45	acyltransferase	1651		KF463149*
4669_IM F2	ATT TCA AAA TTC CCG CTT CC					
pks_bF	ACG GCA TCC TCT GGC ACA TGA C	49			795	KF463150
pks_bR	TCG TCG GAA TCG CAG CTA TGC A					
pks_dF	ACC AGG ACA GAT AAG GAT CGG TG	49	enoyl reductase	430	407	KF463154
pks_dR	AGC GAT GCC TAA CAT TGC CGT A					KF463155
Bmap1_F	CGC CAA AAC CAA CTG TGA CG	53.4	PKS enzyme partial sequences	579	-	KJ210844
Bmar1_R	AAA GGC GGT CCA CCA TC					
KS/AT F	GGA GAT CGC GGC CTG CGT AC	62	3-oxoacyl-(ACP) synthase II	411	417	KJ210845
KS/AT R	GCG ATC TGC GAG ACC CAG CC					KJ210846
KS1 F	TCG ACC CGG ACA CCC TCG AC	65.3	Acetyl CoA acetyltransferase	205	-	KJ210847
KS1 R	AGG GCG ACC CGG ATG GTC T					
R18_F	CGC TTC GGC TCG TCG CTA CC	62	oxidoreductase	421	-	KJ210848
R18_R	TAA CCT CAG CCG CAG GGC GC					
KSAT_F	CGC AAC CGC TGG TTT TGA CGT	49	[acyl-carrier protein] S-malonyl transferase, partial	984	-	KJ210849#
KSAT_R	AGC TAC CGT GCA TTC TGG CTG AC					
KSAT_IM F	GAA TGC ACG GTA GCT ACT TA	45				
KSAT_IM R	TAT GCA AAA ATT TCG CTG CT					
4595_D_N_1	ATT TGA AAG AAC CAA AAC CT	55	Predicted regulatory domain of a methyltransferase	-	399	KJ210850
4595_U_N_1	ATC AAA TGA AAA TGT CTC CGC	55	methyltransferase domain	-	323	KJ2108451
5634_D_N_2	ATA TGG AAG ACG TAA CAT	55	oxidoreductase, β subunit	-	538	KJ2108452

*Both primer pairs contributed for the 1651 bases long acyltransferase fragment from Crimson insects, 4669_IM primers failed to amplify the flanking regions in yellow mutants.

#similarly, both primer pairs amplified overlapping regions of 984 bases long [acyl-carrier protein] S-malonyl transferase domain in crimson insects.

PCR products along with GeneRuler 100 bp Plus (Thermo Scientific, USA) were analyzed on 1% agarose gel (containing ethidium bromide 0.5µg/ml), prepared with 0.5X TBE buffer [45 mM Tris-borate, 1.0 mM EDTA (pH 8.0)] and electrophoresed in 0.5X TBE at 4 Vcm^{-1} for 2 hours. These PCR products were gel purified and sequencing was performed in both directions using the specific primers. In order to verify sequence quality, the analyses were performed with Sequence Scanner v1.0, and good quality sequences having at least 20 QV were subsequently aligned using the online pairwise sequence alignment tool, EMBOSS Needle (Rice *et al.,* 2000) using default parameter values. Nucleotide sequences were analyzed with nucleotide-nucleotide basic local alignment search tool (BLASTn) and Conserved Domains Search tool available at National Center for Biotechnology Information (NCBI) website.

RESULTS AND DISCUSSION

PCR, sequencing and Bioinformatics Analysis

Using the gene specific primers, DNA fragments of acyltransferase domain of PKS ranging approximately 1.6 kb and 0.8 kb was successfully amplified by PCR from total genomic DNA of crimson and yellow lac insects respectively. Also, the partial nucleotide sequences of enoyl reductase of polyketide synthase and 3-oxoacyl-(ACP) synthase II gene were amplified from both colour morphs. However, partial nucleotide sequences of 1.5 kb of [acyl-carrier protein] S-malonyltransferase and 0.5 kb polyketide synthase, were amplified from wild type insects only whereas, the most conserved domain of PKS i.e. β-ketoacyl synthase domain was amplified from the yellow mutant. This 97 bases long β-Ketoacyl synthase fragment of polyketide synthase gene was amplified using degenerate primers 5' AAG CAC ACC TCC MGN CCN TTY GA 3' and 5' ATR YAN YTN CGG GTG CGG TGG CC 3' but could not be submitted to GenBank due to amplicon length limit of 200 bases. Among the post-PKS tailoring enzymes, oxidoreductases were amplified and sequenced from both colour forms but methyltransferase could be amplified only from yellow insects. These primers gave no amplification in negative control (without template) PCR reaction. All the thirteen sequences have been submitted to GenBank (see Table 1 for Accession numbers).

CONCLUSION

Lac insects produce a great variety of metabolites involving a complex set of biosynthetic machinery. The formation of fatty acids and their derivatives, such as hydrocarbons, the polyketides anthraquinones and terpenes are all used by them for various purposes. In insects, pigmentation have been observed to play important roles in behavior such as swarming, feeding (Forsman *et al.*, 2002), social dominance (Tibbetts and Dale, 2004), mate preference, and courtship display (Yeh *et al.*, 2006). It has also been associated with immunity, life-history, physiological, and developmental traits. Insects defend themselves against predators, parasitoids and pathogenic microorganisms. The antimicrobial activity of anthraquinones protects insects from

attack of bacteria and fungi (Cudlin *et al*., 1976; Izhaki, 2002; Kambizi *et al*., 2004; Manojlovic *et al*., 2000). Anthraquinones also show antiviral effects (Barnard *et al*., 1992; Semple *et al*., 2001), as shown by cytostatical activity of carminic acid (Gálvez *et al*., 1996). The anthraquinone present in *Dactylopius confusus* acts as feeding deterrent against ants (Eisner *et al*., 1980). Additionally, avian predators are deterred by several anthraquinones and anthrones (Avery *et al*., 1997; Hilker and Köpf, 1995; Schafer *et al*., 1983). Therefore, in wildlife management, seeds are treated with anthraquinones against avian pests (Avery *et al*., 1997). Brightly coloured polyketidic anthraquinones also find their application in food, cosmetic, paint and dye industries and are commercially valuable antibiotics or pharmacologically active materials contributing to their striking tendency to be developed into therapeutics (a wide range of pharmaceutical activities is achieved by >0.3% of current known polyketides as compared to <0.1% of other natural product or synthetic compounds (Watve *et al*., 2001), others include pigments, or flavoring agents (Hopwood and Sherman, 1990).

This study identifies the multienzyme complex type II polyketide synthase as a key enzyme in *K. lacca* for the biosynthesis of common lac pigment precursor laccaic acid D following polyketide pathway. It appears that both body and resin color pigments are derived through laccaic acid D, which is a component of the body color pigments in wild type crimson insect and only colouring pigment of yellow mutant. Along with the proposal of pathway for biosynthesis of lac constituents, in the present study, we have also identified the partial nucleotide sequences of different enzymes in *K. lacca* such as β-ketoacyl synthase, acyltransferase, enoyl reductase and acyl carrier protein of PKS as well as oxidoreductase and methyl transferase, among post-PKS tailoring enzymes. Whole transcriptome analysis of the *K. lacca* is expected to provide some interesting findings related to PKS and to the enzymes other than PKS which are responsible for producing other constituents of lac dye.

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