tion efficiency of EdDAP and EFdA can be accounted for in terms of different charge distribution effects on the adenine base. This explanation is consistent with experimental results that demonstrated that EdDAP was catabolized more efficiently than EFdA but less efficiently than either dA or EdA, suggesting that a 2-amino substitution has a smaller impact on deamination by ADA than a 2-fluoro substitution (E. N. Kodama, personal communication).

In addition to 2 position substitutions, the presence of a 4′-ethynyl or cyano group also reduces deamination by ADA. For example, ADA deaminates EdA or CNdA less efficiently than dA (Table 4) and EFdA or CNFdA less efficiently than FdA (Fig. 3). Our structural analysis of the predicted interactions of EFdA at the ADA active site suggests that the 4′-ethynyl group may sterically interact with the flexible Met155 side chain (Fig. 4), resulting in a moderate decrease in the binding and reactivity of EFdA compared to the binding and reactivity of dA (Table 4). These data suggest that the effect of 4′-ethynyl on ADA activity is caused primarily by steric interactions. Hence, the substrate specificity of ADA is affected by both electronic and steric interactions from different structural determinants of dA-based NRTIs (2-fluoro and 4′-ethynyl, respectively).

In conclusion, strategic substitutions have a pronounced and complex effect on the ability of dA-based inhibitors to block HIV-1 replication. In particular, substitutions at the 4′ position of the deoxyribose ring and the 2 position of the adenine base of EFdA have separate effects that enhance its antiviral activity by favoring structural conformations that improve its interactions with the RT target and by decreasing its susceptibility to ADA. Because EFdA is a poor substrate for ADA, it is likely to remain intact for longer periods of time, thus reducing the dosage required to achieve an antiviral effect.

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New Susceptibility and Resistance HLA-DP Alleles to HBV-Related Diseases Identified by a Trans-Ethnic Association Study in Asia

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Abstract

Previous studies have revealed the association between SNPs located on human leukocyte antigen (*HLA*) class II genes, including *HLA-DP* and *HLA-DQ*, and chronic hepatitis B virus (HBV) infection, mainly in Asian populations. *HLA-DP* alleles or haplotypes associated with chronic HBV infection or disease progression have not been fully identified in Asian populations. We performed trans-ethnic association analyses of *HLA-DPA1*, *HLA-DPB1* alleles and haplotypes with hepatitis B virus infection and disease progression among Asian populations comprising Japanese, Korean, Hong Kong, and Thai subjects. To assess the association between *HLA-DP* and chronic HBV infection and disease progression, we conducted high-resolution (4-digit) *HLA-DPA1* and *HLA-DPB1* genotyping in a total of 3,167 samples, including HBV patients, HBV-resolved individuals and healthy controls. Trans-ethnic association analyses among Asian populations identified a new risk allele *HLA-DPB1*09:01* ($P = 1.36 \times 10^{-6}$; OR = 1.97; 95% CI, 1.50–2.59) and a new protective allele *DPB1*02:01* ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) to chronic HBV infection, in addition to the previously reported alleles. Moreover, *DPB1*02:01* was also associated with a decreased risk of disease progression in chronic HBV patients among Asian populations ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65). Trans-ethnic association analyses identified Asian-specific associations of *HLA-DP* alleles and haplotypes with HBV infection or disease progression. The present findings will serve as a base for future functional studies of HLA-DP molecules in order to understand the pathogenesis of HBV infection and the development of hepatocellular carcinoma.

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Introduction

Hepatitis B virus (HBV) infection is a major global health problem, resulting in 0.5–1.0 million deaths per year [1]. The prevalence of chronic HBV infection varies. About 75% of the chronic carriers in the world live in Southeast Asia and East Pacific [2]. Due to the introduction of vaccination programs, the prevalence of HBV infection in many countries has gradually been decreasing with consequent decreases in HBV-related hepatocellular carcinoma (HCC) [3]. Although some HBV carriers spontaneously eliminate the virus, about 10–15% of carriers develop liver cirrhosis (LC), liver failure and HCC [4]. Moreover, the progression of liver disease was revealed to be associated with the presence of several distinct mutations in HBV infections [5]. Genetic variations in *STAT4* and *HLA-DQ* genes were recently identified as host genetic factors in a large-scale genome-wide association study (GWAS) for HBV-related HCC in China [6].

With regard to the genes associated with susceptibility to chronic HBV infection, *HLA-DP* and *HLA-DQ* genes were identified by GWAS in Japanese and Thai populations in 2009 [7] and 2011 [8], respectively. In addition, our previous GWAS confirmed and identified the association of SNP markers located on *HLA-DPA1* (rs3077) and *HLA-DPB1* (rs9277535) genes with susceptibility to chronic hepatitis B (CHB) and HBV clearance in Japanese and Korean subjects[9]. The significant associations of *HLA-DP* with CHB and HBV clearance have mainly been detected in Asian populations, such as Japanese [8,9], Thai [7], Chinese [10–12], and Korean [9]. In 2012, the association between *HLA-DPA1* gene SNPs and persistent HBV infection was replicated in a Germany non-Asian population for the first time; however, this showed no association with HBV infection [13]. These results seem to be explained by the fact that allele frequencies of both rs3077 (0.155, 0.587 and 0.743 for C allele, on HapMap CEU, JPT, and YRI) and rs9277535 (0.261, 0.558 and 0.103 for G allele, on HapMap CEU, JPT, and YRI) are markedly different between populations. Moreover, the previous study showed that HBsAg seropositivity rates were higher in Thailand and China (5–12%) than in North America and Europe (0.2–0.5%) [2]. These results suggest that comparative analyses of *HLA-DP* alleles and haplotypes in Asian populations would clarify key host factors of the susceptible and protective *HLA-DP* alleles and haplotypes for CHB and HBV clearance. Here, we performed trans-ethnic analyses of *HLA-DP* alleles and haplotypes in Asian populations comprising Japanese, Korean, Hong Kong and Thai individuals. The findings from this study will serve as a base for future functional studies of HLA-DP molecules.

Results

Characteristics of studied subjects

The characteristics of a total of 3,167 samples, including Japanese, Korean, Hong Kong and Thai subjects, are shown in Table 1. Each population included three groups of HBV patients, resolved individuals and healthy controls. The clinical definitions of HBV patients and resolved individuals are summarized in Materials and Methods. Some of the Japanese and all of the Korean samples overlapped with the subjects in our previous study [9,14].

We performed genotyping for *HLA-DPA1* and *HLA-DPB1* in all 3,167 samples, and a total of 2,895 samples were successfully genotyped. The characteristics of successfully genotyped samples are shown in Table S1.

Association of *HLA-DPA1* and *HLA-DPB1* alleles in Asian populations

As for a general Asian population, including 464 Japanese, 140 Korean, 156 Hong Kong, and 122 Thai subjects, five *HLA-DPA1* alleles and twenty-four *HLA-DPB1* alleles were observed (Table S2). The frequencies of *HLA-DPA1* and *HLA-DPB1* alleles were similar between Japanese and Korean subjects. On the other hand, the number of alleles with frequencies of 1–2% was larger in Hong Kong and Thai populations, despite the small sample size. Although the frequencies of *HLA-DP* alleles varied in Asian populations, *HLA-DPB1*05:01* was the most prevalent with over 30% in all populations.

The associations of *HLA-DPA1* and *HLA-DPB1* alleles with chronic HBV infection (i.e., comparison between HBV patients and healthy controls) are shown in Table S2. To avoid false positives caused by multiple testing, the significance levels were corrected based on the numbers of *HLA-DPA1* and *HLA-DPB1*

Table 1. Number of individuals in this study.

Population	Japanese	Korean	Hong Kong	Thai
Total number of samples	1,291	586	661	629
HBV patients	489	340	281	390
IC	114	-	-	-
CH	147	175	187	198
AE	21	-	-	-
LC	38	-	-	-
HCC	169	165	94	192
Mean age (y)	57.1	44.7	57.9	52.0
(min-max)	(20–84)	(18–74)	(32–86)	(21–84)
Gender (M/F)	338/151	265/75	239/42	289/101
Resolved individuals*	335	106	190	113
HCV (−)	249	106	190	113
HCV (+)	86	-	-	-
Mean age (y)	59.7	43.1	40.0	48.2
(min-max)	(18–87)	(12–66)	(18–60)	(39–66)
Gender (M/F)	173/162	61/45	113/77	83/30
Healthy controls	467	140	190	126
Mean age (y)	39.0**	33.7	26.2	46.6
(min-max)	(23–64)	(1–59)	(16–60)	(38–79)
Gender (M/F)	370/97	67/73	87/103	73/53

Abbreviation: IC, Inactive Carrier; CH, Chronic Hepatitis; AE, Acute Exacerbation; LC, Liver Cirrhosis; HCC, Hepatocellular Carcinoma.

* Resolved individuals were HBsAg negative and HBcAb positive.

** 419 of 467 healthy controls were de-identified, without information on age.

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alleles in the focal population. Briefly, the significance level was set at 0.05/(# of observed alleles at each locus) in each population (see Materials and Methods). With regard to high-risk alleles of *HLA-DPA1*, the most prevalent allele *HLA-DPA1*02:02* was significantly associated with susceptibility to HBV infection in Japanese ($P = 3.45 \times 10^{-4}$; OR = 1.39; 95% CI, 1.16–1.68) and Korean subjects ($P = 2.66 \times 10^{-5}$; OR = 1.89; 95% CI, 1.39–2.58), whereas this association was not observed in Hong Kong or Thai subjects. The association of *HLA-DPA1*02:01* with susceptibility to HBV infection was significant only in Japanese ($P = 2.61 \times 10^{-7}$; OR = 1.88; 95% CI, 1.46–2.41). The significant association of *HLA-DPA1*01:03* with protection against HBV infection was commonly observed among four Asian populations (Table S2). The pooled OR and 95% CI were 0.51 and 0.41–0.63, respectively in a meta-analysis ($P = 3.15 \times 10^{-10}$) (Fig. S1A).

As shown in Table S2, *HLA-DPB1* shows higher degree of polymorphism than *HLA-DPA1*. The most common allele in Asian populations, *HLA-DPB1*05:01*, was significantly associated with HBV susceptibility in both Japanese and Korean subjects. Although *HLA-DPB1*05:01* showed no significant association in the Hong Kong and Thai populations, the same direction of association (i.e., HBV susceptibility) was observed. Meta-analysis of the four populations revealed a significant association between *HLA-DPB1*05:01* and susceptibility to HBV infection ($P = 1.51 \times 10^{-4}$; OR = 1.45; 95% CI, 1.19–1.75) (Fig. S1B). The frequency of *HLA-DPB1*09:01* was significantly elevated in Japanese HBV patients (15.7%) as compared with healthy controls (8.7%) ($P = 3.70 \times 10^{-6}$; OR = 1.94; 95% CI, 1.45–2.62), and this association was most significant (i.e., the smallest P value) in the Japanese population. Because of lower allele frequencies of *HLA-DPB1*09:01* or lack of statistical power in the other populations, no significant associations were observed. A common allele in Thai subjects, *HLA-DPB1*13:01*, was significantly associated with susceptibility to HBV infection ($P = 2.49 \times 10^{-4}$; OR = 2.17; 95% CI, 1.40–3.47) with the same direction of associations in Japanese and Hong Kong (OR = 1.52 and 1.40, respectively).

*HLA-DPB1*04:02* was identified as the most protective allele for HBV infection in Japanese ($P = 1.59 \times 10^{-7}$; OR = 0.37; 95% CI, 0.24–0.55) and Korean subjects ($P = 1.27 \times 10^{-7}$; OR = 0.19; 95% CI, 0.10–0.38). Both *HLA-DPB1*02:01* and *HLA-DPB1*04:01* were also significantly associated with protection in the Japanese population, and the former was significantly associated with protection in Hong Kong subjects ($P = 9.17 \times 10^{-4}$; OR = 0.49; 95% CI, 0.32–0.76). This common allele among four Asian populations, *HLA-DPB1*02:01*, showed a significant association with protection against HBV infection ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) in a meta-analysis (Fig. S1B).

The frequencies of associated *HLA-DP* alleles in a comparison of HBV patients with healthy controls (Table S2) or with HBV-resolved individuals (Table S3) were similar in all four Asian populations. In the Japanese population, the associations of susceptible and protective *HLA-DPB1* alleles to chronic HBV infection seem weaker in the comparison of HBV patients with HBV-resolved individuals than in the comparison of HBV patients with healthy controls. Moreover, the results of association analyses showed no difference in the comparison of HBV patients with HBV-resolved individuals, including or excluding HCV positive individuals (Table S3). In contrast, the association became stronger in the comparison of HBV patients with HBV-resolved individuals among the Korean subjects. The protective allele *HLA-DPB1*04:01* was also identified to have a strong association with HBV clearance in Hong Kong subjects (Table S3). Moreover, in Hong Kong subjects, the *HLA-DPB1*05:01* associated with the risk for HBV infection showed lower frequency in HBV-resolved individuals (42.9%) than in the healthy controls (48.1%), which accounts for a strong association in the comparison of HBV patients with HBV-resolved individuals ($P = 6.24 \times 10^{-3}$; OR = 1.64; 95% CI, 1.14–2.36). Although the number of samples was insufficient, *HLA-DP*100:01* showed a significant association with protection against HBV infection in the Hong Kong population ($P = 3.05 \times 10^{-6}$; OR = 0.03; 95% CI, 0.0007–0.20).

Table 2. Association of number of *DPB1*02:01* alleles (i.e., 0, 1 or 2) with disease progression in CHB patients assessed by multivariate logistic regression analysis adjusted for age and sex.

Population	P value	OR (95% CI)
Japanese	0.000177	0.47 (0.32–0.70)
Korean	0.025358	0.55 (0.33–0.93)
Hong Kong	0.040842	0.46 (0.22–0.97)
Thai	0.087782	0.58 (1.31–1.08)
All*	1.55×10^{-7}	0.50 (0.39–0.65)

*Population was adjusted using dummy variables.

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As for disease progression in CHB patients among Asian populations, a protective effect of *HLA-DPB1*02:01* on disease progression was observed in the Japanese ($P = 4.26 \times 10^{-5}$; OR = 0.45; 95% CI, 0.30–0.67) and Korean populations ($P = 8.74 \times 10^{-4}$; OR = 0.47; 95% CI, 0.29–0.75) (Table S4). Multivariate logistic regression analysis adjusted for age and sex revealed that the number of *DPB1*02:01* alleles (i.e., 0, 1, or 2) was significantly associated with disease progression in CHB patients in Japanese ($P = 1.77 \times 10^{-4}$; OR = 0.47; 95% CI, 0.32–0.70) (Table 2). Moreover, protective effects of *DPB1*02:01* on disease progression in Asian populations ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65) were detected in a multivariate logistic regression analysis adjusted for age, gender, and population (Table 2).

Associations of *DPA1-DPB1* haplotypes in Asian populations

The estimated frequencies of *HLA DPA1-DPB1* haplotypes are shown in Table S5. The most frequent haplotype among the four Asian populations was *DPA1*02:02-DPB1*05:01*. The number of haplotypes with low frequencies of 1–2% was 10 in both Japanese and Korean subjects, whereas more haplotypes appeared with frequencies of 1–2% in Hong Kong and Thai subjects. The associations of *DPA1-DPB1* haplotypes with HBV infection are shown in Table S5. In the Japanese population, *DPA1*02:01-DPB1*09:01* showed the most significant association with susceptibility to HBV infection ($P = 3.38 \times 10^{-6}$; OR = 1.95; 95% CI, 1.46–2.64). The most common haplotype in the four Asian populations, *DPA1*02:02-DPB1*05:01*, was found to be significantly associated with susceptibility to HBV infection in the Japanese and Korean subjects ($P = 7.40 \times 10^{-4}$; OR = 1.37; 95% CI, 1.14–1.66 for Japanese, and $P = 4.50 \times 10^{-6}$; OR = 2.02; 95% CI, 1.48–2.78 for Korean). In the Thai subjects, *HLA-DPB1*13:01* was the most significant risk allele for HBV infection (Table S2); however, no significant associations were found for the three different haplotypes bearing *HLA-DPB1*13:01*: *DPA1*02:01-DPB1*13:01*, *DPA1*02:02-DPB1*13:01*, and *DPA1*04:01-DPB1*13:01*,indicating that the association of *HLA-DPB1*13:01* with susceptibility to HBV infection did not result from a specific *DPA1-DPB1* haplotype or combination with a specific *DPA1* allele.

In the Japanese population, both haplotypes *DPA1*01:03-DPB1*04:01* and *DPA1*01:03-DPB1*04:02* showed significant associations with protection against HBV infection ($P = 1.17 \times 10^{-5}$; OR = 0.32; 95% CI, 0.18–0.56 for *DPA1*01:03-DPB1*04:01* and $P = 1.95 \times 10^{-7}$; OR = 0.37; 95% CI, 0.24–0.55 for *DPA1*01:03-DPB1*04:02*). In the Korean subjects, a significant association of *DPA1*01:03-DPB1*04:02* was also demonstrated; however, no association was observed for *DPA1*01:03-DPB1*04:01*. Because the observed number of each haplotype was small, none of the other haplotypes showed a significant association with protection against HBV infection.

In order to identify trans-ethnic DPA1-DPB1 haplotypes associated with HBV infection, a meta-analysis was performed. A meta-analysis further revealed that the *DPA1*01:03-DPB1*02:01* haplotype was significantly associated with protection against HBV infection ($P = 1.45 \times 10^{-5}$; OR = 0.69; 95% CI, 0.58–0.82) (Fig. S1C).

Discussion

Among 2.2 billion individuals worldwide who are infected with HBV, 15% of these are chronic carriers. Of chronic carriers, 10–15% develops LC, liver failure and HCC, and the remaining individuals eventually achieve a state of nonreplicative infection, resulting in HBsAg negative and anti-HBc positive, i.e. HBV-resolved individuals. To identify host genetic factors associated with HBV-related disease progression may lead HBV patients to discriminate individuals who need treatment.

The *HLA-DPA1* and *HLA-DPB1* genes were identified as host genetic factors significantly associated with CHB infection, mainly in Asian populations [7–12], and not in European populations [13]. In the previous association analyses of *HLA-DPB1* alleles with HBV infection, one risk allele *HLA-DPB1*05:01* (OR = 1.52; 95% CI, 1.31–1.76), and two protective alleles, *HLA-DPB1*04:01* (OR = 0.53; 95% CI, 0.34–0.80) and *HLA-DPB1*04:02* (OR = 0.47; 95% CI, 0.34–.64), were identified in the Japanese population [7]. In this study, we further identified a new risk allele *HLA-DPB1*09:01* (OR = 1.94; 95% CI, 1.45–2.62) for HBV infection and a new protective allele *HLA-DPB1*02:01* (OR = 0.71; 95% CI, 0.56–0.89) in the Japanese population, in addition to the previously reported alleles (Table S2) [7]. The discrepancy in the association of *HLA-DPB1*09:01* allele with risk for HBV infection in a previous study [7] results from the elevated frequency of *HLA-DPB1*09:01* in the controls (12.2%), which is higher than our controls (8.7%). In this study, healthy subjects were recruited as controls. In contrast, individuals that were registered in BioBank Japan as subjects with diseases other than CHB were recruited as controls in the previous study [7], which may have included patients with diseases with which *HLA-DPB1*09:01* is associated. Although no significant association of *HLA-DPB1*09:01* with risk for HBV infection was observed in the Korean subjects, *HLA-DPB1*09:01* appears to have a susceptible effect on HBV infection, as it showed the same direction of association. When the association analyses in Japanese and Korean subjects were combined in meta-analysis, the association was statistically significant ($P = 1.36 \times 10^{-6}$; OR = 1.97; 95% CI, 1.50–2.59). Thus, *HLA-DPB1*09:01* may be a Northeast Asian-specific allele associated with risk for HBV infection.

Moreover, a significant association of *HLA-DPB1*13:01* with risk of HBV infection (OR = 2.17; 95% CI, 1.40–3.47) was identified in the Thai subjects. However, the frequency of *HLA-DPB1*13:01* in Thai healthy controls (11.5% in the present study) reportedly varies, ranging from 15.4% to 29.5%, due to the population diversity [15–17]. Therefore, a replication analysis is required to confirm the association of *HLA-DPB1*13:01* with HBV infection in the Thai subjects. There were four other marginally associated *HLA-DPB1* alleles with low allele frequencies below 5% in HBV patients and healthy controls, including *HLA-DPB1*28:01*, *-DPB1*31:01*, *-DPB1*100:01*, and *-DPB1*105:01*, in the Hong Kong and Thai subjects. Because these infrequent alleles may have resulted from false positive associations, the association needs to be validated in a large number of subjects.

*HLA-DPB1*02:01* showed a significant association with protection against HBV infection in both Japanese and Hong Kong populations (Table S2); however, the *HLA-DPB1*02:01* allele was not associated with HBV infection in the previous study [7]. Although *HLA-DPB1*02:01* showed no association in either Korean or Thai populations, a significant association of *HLA-DPB1*02:01* with protection against HBV infection among four Asian populations was detected in meta-analysis ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) (Fig. S1B). We therefore conclude that the present finding is not a false positive.

A recent report showed that *HLA-DPB1*02:01:02*, **02:02*, **03:01:01*, **04:01:01*, **05:01*, **09:01*, and **14:01* were significantly associated with response to booster HB vaccination in Taiwan neonatally vaccinated adolescents [18]. The *HLA-DPB1*02:01:02*, **02:02*, **03:01:01*, **04:01:01*, and **14:01* were significantly more frequent in recipients whose post-booster titers of antibodies against HBV surface antigen (anti-HBs) were detectable, on the other hand, *HLA-DPB1*05:01* and **09:01* were significantly more frequent in recipients who were undetectable. Moreover, the *HLA-DPB1*05:01* and **09:01* significantly increase the likelihoods of undetectable pre-booster anti-HBs titers. These results seem consistent with our findings, in which *HLA-DPB1*05:01* and **09:01* are associated with susceptibility to chronic hepatitis B infection.

We also identified a protective effect of *HLA-DPB1*02:01* allele on disease progression in Asian populations. Previous studies identified the association of HLA class II genes including *HLA-DQ* and *HLA-DR* with development of HBV related hepatocellular carcinoma in the Chinese population [6,19,20]. In this study using Japanese and Korean samples, we identified significant associations between *HLA-DPB1*02:01* and disease progression in CHB patients ($P = 4.26 \times 10^{-5}$; OR = 0.45; 95% CI, 0.30–0.67, for Japanese and $P = 8.74 \times 10^{-4}$; OR = 0.47; 95% CI, 0.29–0.75 for Korean) (Table S4). Although the association of *HLA-DPB1*02:01* with disease progression was weaker after adjustment for age and gender in Korean subjects ($P = 2.54 \times 10^{-2}$; OR = 0.55; 95% CI, 0.33–0.93), the same direction of association was observed (i.e. protective effect on disease progression) (Table 2). The protective effects of *HLA-DPB1*02:01* on disease progression showed a significant association after adjustment for age and gender in the Japanese population ($P = 1.77 \times 10^{-4}$; OR = 0.47; 95% CI, 0.32–0.70); moreover, a significant association between *HLA-DPB1*02:01* was observed among four Asian populations, under which population was adjusted by using dummy variables in a multivariate logistic regression analysis ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65) (Table 2).

The *HLA-DPA1* and *HLA-DPB1* belong to the HLA class II alpha and beta chain paralogues, which make a heterodimer consisting of an alpha and a beta chain on the surface of antigen presenting cells. This HLA class II molecule plays a central role in the immune system by presenting peptides derived from extracellular proteins. We identified two susceptible haplotypes (*DPA1*02:02-DPB1*05:01* and *DPA1*02:01-DPB1*09:01*) and three protective haplotypes (*DPA1*01:03-DPB1*04:01*, *DPA1*01:03-DPB1*04:02*, and *HLA-DPA1*01:03-DPB1*02:01*) to chronic hepatitis B infection, which may result in different binding

affinities between HLA-DP subtypes and extracellular antigens. Although functional analyses of HLA-DP subtypes to identify HBV-related peptides are not fully completed, identification of susceptible and protective haplotypes as host genetic factors would lead us to understand the pathogenesis of HBV infection including viral factors.

In summary, we identified a new risk allele *HLA-DPB1*09:01*, which was specifically observed in Northeast Asian populations, Japanese and Korean. Moreover, a new protective allele *HLA-DPB1*02:01* was identified among four Asian populations: Japanese, Korean, Hong Kong and Thai. The protective allele *HLA-DPB1*02:01* was associated with both chronic HBV infection and disease progression in chronic HBV patients. Identification of a total of five alleles, including two risk alleles (*DPB1*09:01* and *DPB1*05:01*) and three protective alleles (*DPB1*04:01*, *DPB1*04:02* and *DPB1*02:01*), would enable HBV-infected individuals to be classified into groups according to the treatment requirements. Moreover, the risk and protective alleles for HBV infection and disease progression, identified in this study by means of trans-ethnic association analyses, would be key host factors to recognize HBV-derived antigen peptides. The present results may lead to subsequent functional studies into HLA-DP molecules and viral factors in order to understand the pathogenesis of HBV infection and development of hepatocellular carcinoma.

Materials and Methods

Ethics Statement

All study protocols conform to the relevant ethical guidelines, as reflected in the *a priori* approval by the ethics committee of National Center for Global Health and Medicine, and by the ethics committees of all participating universities and hospitals, including The University of Tokyo, Japanese Red Cross Kanto-Koshinetsu Block Blood Center, The University of Hong Kong, Chulalongkorn University, Yonsei University College of Medicine, Nagoya City University Graduate School of Medical Sciences, Musashino Red Cross Hospital, Tokyo Medical and Dental University, Teine Keijinkai Hospital, Hokkaido University Graduate School of Medicine, Kurume University School of Medicine, Okayama University Graduate School of Medicine, Yamaguchi University Graduate School of Medicine, Tottori University, Kyoto Prefectural University of Medicine, Osaka City University Graduate School of Medicine, Nagoya Daini Red Cross Hospital, Ehime University Graduate School of Medicine, Kanazawa University Graduate School of Medicine, National Hospital Organization Osaka National Hospital, Iwate Medical University, Kawasaki Medical College, Shinshu University School of Medicine, Saitama Medical University, Kitasato University School of Medicine, Saga Medical School, and University of Tsukuba.

Written informed consent was obtained from each patient who participated in this study and all samples were anonymized. For Japanese healthy controls, 419 individuals were de-identified with information about gender, and all were recruited after obtaining verbal informed consent in Tokyo prior to 1990. For the 419 Japanese healthy individuals, written informed consent was not obtained because the blood sampling was conducted before the "Ethical Guidelines for Human Genome and Genetic Sequencing Research" were established in Japan. Under the condition that DNA sample is permanently de-linked from the individual, this study was approved by the Research Ethics Committee of National Center for Global Health and Medicine.

Characteristics of studied subjects

All of the 3,167 genomic DNA samples were collected from individuals with HBV, HBV-resolved individuals (HBsAg-negative and anti-HBc-positive) and healthy controls at 26 multi-center hospitals throughout Japan, Korea, Hong Kong, and Thailand (Table 1). In a total of 1,291 Japanese and 586 Korean samples, 1,191 Japanese individuals and all 586 Korean individuals were included in our previous study [9]. With regard to additional Japanese individuals, we collected samples from 48 healthy controls at Kohnodai Hospital, and 52 HBV patients at Okayama University Hospital and Ehime University Hospital, including 26 individuals with LC and 26 individuals with HCC. A total of 661 Hong Kong samples and 629 Thai samples were collected at Queen Mary Hospital and Chulalongkorn University, respectively.

HBV status was measured based on serological results for HBsAg and anti-HBc with a fully automated chemiluminescent enzyme immunoassay system (Abbott ARCHITECT; Abbott Japan, Tokyo, Japan, or LUMIPULSE f or G1200; Fujirebio, Inc., Tokyo, Japan). For clinical staging, inactive carrier (IC) state was defined by the presence of HBsAg with normal ALT levels over 1 year (examined at least four times at 3-month intervals) and without evidence of liver cirrhosis. Chronic hepatitis (CH) was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/L]) persisting over 6 months (by at least 3 bimonthly tests). Acute exacerbation (AE) of chronic hepatitis B was defined as an elevation of ALT to more than 10 times the upper limit of normal (ULN, 58 IU/L) and bilirubin to at least three times ULN (15 μmol/L). LC was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges and hypersplenism), platelet counts<100,000/cm^3, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. HCC was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy or a combination thereof.

The Japanese control samples from HBV-resolved subjects (HBsAg-negative and anti-HBc-positive) at Nagoya City University-affiliated healthcare center were used by comprehensive agreement (anonymization in a de-identified manner) in this study. Some of the unrelated and anonymized Japanese healthy controls were purchased from the Japan Health Science Research Resources Bank (Osaka, Japan). One microgram of purified genomic DNA was dissolved in 100 μl of TE buffer (pH 8.0) (Wako, Osaka, Japan), followed by storage at −20°C until use.

Genotyping of *HLA-DPA1* and *HLA-DPB1* alleles

High resolution (4-digit) genotyping of *HLA-DPA1* and *-DPB1* alleles was performed for HBV patients, resolved individuals, and healthy controls in Japan, Korea, Hong Kong, and Thailand. LABType SSO HLA DPA1/DPB1 kit (One Lambda, CA) and a Luminex Multi-Analyte Profiling system (xMAP; Luminex, Austin, TX) were used for genotyping, in according with the manufacturer's protocol. Because of the small quantity of genomic DNA in some Korean samples, we performed whole genome amplification for a total of 486 samples using GenomiPhi v2 DNA Amplification kit (GE Healthcare Life Sciences, UK), in accordance with the manufacturer's instruction.

A total of 2,895 samples were successfully genotyped and characteristics of these samples are summarized in Table S1.

Statistical analysis

Fisher's exact test in two-by-two cross tables was used to examine the associations between *HLA-DP* allele and chronic HBV infection or disease progression in chronic HBV patients,

using statistical software R2.9. To avoid false-positive results due to multiple testing, significance levels were adjusted based on the number of observed alleles at each locus in each population. For *HLA-DPA1* alleles, the number of observed alleles was 3 in Japanese, 4 in Korean, 5 in Hong Kong, and 5 in Thai subjects. Therefore, the significant levels for α were set at $\alpha = 0.05/3$ in Japanese, $\alpha = 0.05/4$ in Korean, $\alpha = 0.05/5$ in Hong Kong, and $\alpha = 0.05/5$ in Thai subjects. In the same way, significant levels for *HLA-DPB1* alleles were $\alpha = 0.05/10$, $0.05/11$, $0.05/12$, and $0.05/16$, respectively. Multivariate logistic regression analysis adjusted for age and sex (used as independent variables) was applied to assess associations between the number of *DPB1*02:01* alleles (i.e., 0, 1, or 2) and disease progression in CHB patients. To examine the effect of *DPB1*02:01* allele on disease progression in all populations, population was further adjusted by using three dummy variables (i.e., (c1, c2, c3) = (0, 0, 0) for Japanese, (1, 0, 0) for Korean, (0, 1, 0) for Hong Kong, and (0, 0, 1) for Thai) in a multivariate logistic regression analysis. We obtained the following regression equation: $\text{logit}(p) = -3.905 + 0.083 \times \text{age} + (-0.929) \times \text{sex} + (-0.684) \times \textit{DPB1*02:01} + 1.814 \times c1 + (-0.478) \times c2 + 0.782 \times c3$. Significance levels in the analysis of disease progression in CHB patients were set as $\alpha = 0.05/10$ in Japanese, $\alpha = 0.05/11$ in Korean, $\alpha = 0.05/15$ in Hong Kong, and $\alpha = 0.05/15$ in Thai subjects. The phase of each individual (i.e., a combination of two *DPA1-DPB1* haplotypes) was estimated using PHASE software [21], assuming samples are selected randomly from a general population. In comparison of the estimated *DPA1-DPB1* haplotype frequencies, significant levels were set as $\alpha = 0.05/14$ in Japanese, $\alpha = 0.05/17$ in Korean, $\alpha = 0.05/17$ in Hong Kong, and $\alpha = 0.05/18$ in Thai subjects. Meta-analysis was performed using the DerSimonian-Laird method (random-effects model) in order to calculate pooled OR and its 95% confidence interval (95% CI). We applied meta-analysis for alleles with frequency>1% in all four Asian populations. The significance levels in meta-analysis were adjusted by the total number of statistical tests; $\alpha = 0.05/20$ for *DPA1* alleles, $\alpha = 0.05/57$ for *DPB1* alleles, and $\alpha = 0.05/74$ for *DPA1-DPB1* haplotypes.

Supporting Information

Figure S1 Comparison of odds ratios in association analyses for *HLA-DP* with chronic HBV infection among four Asian populations: (A) *HLA-DPA1* alleles; (B) *HLA-DPB1* alleles; and (C) *HLA DPA1-DPB1* haplotypes. Meta-analysis was performed using the DerSimonian-Laird method (random-effects model) to calculate pooled OR and its 95% confidence interval (95% CI). Bold depicts a statistically significant association after correction of significance level.
(DOCX)

Table S1 Individuals with successfully genotyped for *HLA-DPA1* and *HLA-DPB1*.
(DOCX)

Table S2 Frequencies of HLA-DP alleles in HBV patients and healthy controls among Asian populations.
(XLSX)

Table S3 Frequencies of HLA-DP alleles in HBV patients and resolved individuals among Asian populations.
(XLSX)

Table S4 Associations of HLA-DPB1 alleles with disease progression in CHB patients among Asian populations.
(XLSX)

Table S5 Estimated frequencies of HLA DPA1-DPB1 haplotypes in HBV patients and healthy controls among Asian populations.
(XLSX)

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Author Contributions

Conceived and designed the experiments: NN HS MS KT M. Mizokami. Performed the experiments: NN HS KK Y. Mawatari M. Kawashima M. Minami. Analyzed the data: NN HS M. Kawashima JO. Contributed reagents/materials/analysis tools: W-KS M-FY NP YP SHA K-HH K. Matsuura YT M. Kurosaki YA NI J-HK SH TI KY IS Y. Murawaki YI AT EO YH MH SK EM KS KH ET SM MW YE NM K. Murata M. Korenaga KT M. Mizokami. Wrote the paper: NN HS JO KT M. Mizokami.

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Cloning and Heterologous Expression of the Aurachin RE Biosynthesis Gene Cluster Afford a New Cytochrome P450 for Quinoline N-Hydroxylation

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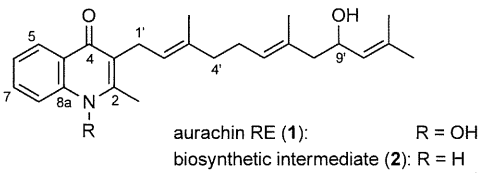
Aurachin RE is a prenylated quinoline antibiotic that was first isolated from the genus *Rhodococcus*. It shows potent antibacterial activity against a variety of Gram-positive bacteria. Here we have identified a minimal biosynthesis gene cluster for aurachin RE in *Rhodococcus erythropolis* JCM 6824 by using random transposon mutagenesis and heterologous production. The *Rhodococcus* aurachin (*rau*) gene cluster consists of genes encoding cytochrome P450 (*rauA*), prenyltransferase, polyketide synthase, and farnesyl pyrophosphate synthase, as well as others including genes involved in regulation and transport. Markerless gene disruption of *rauA* resulted in the complete loss of aurachin RE production and in the accumulation of a new aurachin derivative lacking the N-hydroxy group. When the recombinant RauA was expressed in *Escherichia coli*, it catalyzed N-hydroxylation of the derivative to form aurachin RE. This study establishes the biosynthetic pathway of aurachin RE and provides experimental evidence for the role of P450 RauA in catalyzing N-hydroxylation of the quinoline ring, which is indispensable for the antibacterial activity of aurachin RE.

Introduction

The genus *Rhodococcus* is made up of a diverse group of bacteria that are commonly found in a broad range of environments such as soil and seawater. These Gram-positive coryneform bacteria have high G+C contents and belong to the order *Actinomycetales*. Many *Rhodococcus* strains show remarkable metabolic versatility, as exemplified by their ability to degrade a variety of persistent xenobiotics such as polychlorinated biphenyls.[1] Additionally, engineered *Rhodococcus* strains are used as an alternative host microorganism for heterologous protein production.[2] Significant knowledge about *Rhodococcus* has therefore been acquired, and the genetic tools for this genus are highly developed. Despite these remarkable advances, however, reports describing antibacterial compounds isolated from *Rhodococcus* are limited,[3] although a few antimicrobial peptides such as rhodopeptins and lariatins have been shown to be produced by this genus.[4]

We have previously identified 15 different *Rhodococcus erythropolis* strains that produce unidentified compounds that exhibit antibacterial activity; they have been classified into three groups (groups I, II, and III) according to their antibacterial spectra. Group I strains exhibited growth inhibition activity against a broad range of Gram-positive bacteria. Group II strains exhibited growth inhibition activity mainly against the genus *Rhodococcus* and some other Gram-positive bacteria. Group III strains exhibited growth inhibition activity particularly against *R. erythropolis*.[3]

We had previously isolated a new quinoline antibiotic—aurachin RE (**1**, Scheme 1), classified as group I—from a culture broth of *R. erythropolis* JCM 6824.[5] The structure of an aurachin basically consists of a quinolone ring and a farnesyl chain, and the structure of aurachin RE is closely related to that of aurachin C (H instead of 9′-OH), which was isolated from *Stigmatella aurantiaca* Sg a15.[6] Aurachins and related compounds have been found to be potent inhibitors of mitochondrial respiration through blockage of NADH oxidation, presumably thanks to their significant structural similarity to vitamin K.[7]



Scheme 1. Chemical structures of aurachin RE (**1**) and its biosynthetic intermediate **2**.

Aurachin alkaloids were originally isolated from the Gram-negative bacterium *Stigmatella* in the 1980s. Interestingly, only

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one producer species other than *Stigmatella*, the Gram-positive bacterium *R. erythropolis*, has been identified subsequently. It is particularly interesting that two phylogenetically unrelated species of microorganisms produce such highly structurally related secondary metabolites. Although aurachin biosynthesis genes (*aua* genes) have been identified in *Stigmatella*,[8] our preliminary PCR screening study, based on the nucleotide and amino acid (aa) sequence data for *aua*, failed to identify the corresponding genes in the *Rhodococcus* genome.[5] These results suggested that this *Rhodococcus* strain does not contain *aua*-type biosynthesis genes or biosynthesis genes similar to those that were isolated from *Stigmatella*.

In this study we aimed to isolate aurachin alkaloid biosynthesis genes in *Rhodococcus* by the transposon mutagenesis method, in order to characterize the biosynthetic pathway and to determine the evolutionary relationships between *aua* genes. Here we describe the identification of *Rhodococcus* aurachin biosynthesis genes (*rau* genes). The functions of *rau* genes were analyzed by targeted gene mutagenesis, followed by gene complementation tests and heterologous expression analyses. Notably, N-hydroxylation of the quinoline ring was found to be catalyzed by a new P450 monooxygenase (RauA), which is indispensable for the antibacterial activity of aurachin RE.

Results

Isolation of the aurachin RE biosynthesis genes

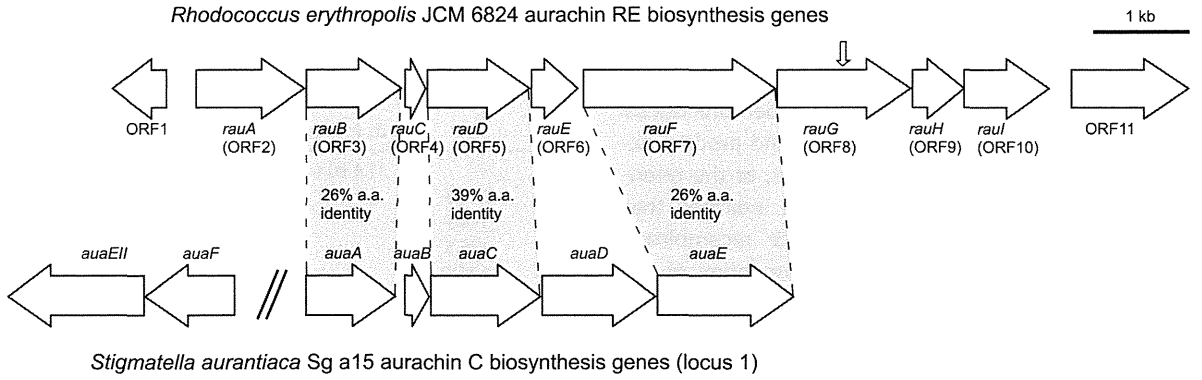
In order to identify the biosynthesis genes of aurachin RE in *Rhodococcus*, an antibiotic-deficient transposon mutant of JCM 6824 was isolated. Random transposon mutagenesis was carried out by use of the suicide transposon vector pTNR.[9] The vector was introduced into wild-type cells by electroporation, and the kanamycin-resistant mutants generated were then screened for the loss of antibiotic activity. Consequently, an antibiotic-activity-deficient clone was obtained from 2000 mutant clones. The mutant strain, M18-8, had completely lost its activity against the indicator *Arthrobacter* strain.

To isolate the aurachin biosynthesis genes, nucleotide sequences flanking the transposable element in the M18-8 strain's genome were analyzed by the plasmid rescue method.[9] In combination with nucleotide sequence analysis of the cosmid DNA library of the wild-type JCM 6824 genome, it was possible to determine 17525 bp of the nucleotide sequence containing the region. As shown in Table 1 and Scheme 2, the sequence contained 11 open reading frames (ORFs), and the introduced transposable element was located on the eighth ORF in the mutant strain. Some of the deduced amino acid sequences showed similarity to those of the gene products of polyketide-synthesis-related enzymes (type II, ORFs 4, 5, 6) or terpenoid-synthesis-related enzymes (ORFs 9 and 10). Because the structure of aurachin RE contains both quinoline and sesquiterpene moieties, it was expected that these genes would be involved in the production of the antibiotics. The data for these genes showed that the deduced amino acid sequences of ORFs 3, 5, and 7 showed some similarity with the aurachin C biosynthesis genes (*aua*) of *S. aurantiaca* Sga15:[8] ORF3 showed 26% amino acid identity with AuaA prenyl-transferase, ORF5 showed 39% identity with AuaC β-ketoacyl ACP synthase, and ORF7 showed 26 and 27% identity with AuaE anthranilate-CoA-ACP transferase and AuaEII anthranilate-CoA ligase, respectively. The other ORFs—that is, 1, 2, and 8—showed similarity with a TetR-type transcriptional regulator, cytochrome P450 monooxygenase, and a major facilitator superfamily (MFS) transporter, respectively (Table 1).

The deduced protein sequence of ORF11 did not show significant similarity with any protein in the databases. We inferred from these results that these ORFs were the *Rhodococcus* aurachin RE biosynthesis genes and designated ORF2–ORF10 as *rauA–I*, respectively (Table 1 and Scheme 2). The sequence similarities described above indicated that *rauB*, *-C*, *-D*, *-E*, *-F*, *-H*, and *-I* are core biosynthesis genes for the production of aurachin RE.

Table 1. Deduced functions of ORFs in the aurachin RE biosynthesis gene cluster (deduced amino acid sequence).

ORF (protein encoded)	Size [aa]	Protein homologue, origin (accession no.)	Identity/ similarity [%]	Deduced function
ORF1	193	SCO0646, *Streptomyces coelicolor* A3(2) (NP_624955)	35/56	transcriptional regulator, TetR type
ORF2 (RauA)	411	Mjls_5328, *Mycobacterium* sp. JLS (YP_001073582)	34/50	cytochrome P450 monooxygenase
ORF3 (RauB)	353	AuaA, *Stigmatella aurantiaca* Sg a15 (CAL48953)	26/45	prenyltansferase (farnesyl transferase)
ORF4 (RauC)	82	ABSDF2639, *Acinetobacter baumannii* SDF (YP_001707848)	33/63	acyl carrier protein (ACP)
ORF5 (RauD)	387	AuaC, *Stigmatella aurantiaca* Sg a15 (CAL48955)	39/56	β-ketoacyl ACP synthase II
ORF6 (RauE)	176	SGR_1344, *Streptomyces griseus* NBRC 13350 (YP_001822856)	30/44	β-ketoacyl ACP synthase II
ORF7 (RauF)	707	AuaE and AuaEII, *Stigmatella aurantiaca* Sg a15 (CAL48957 and CCA65703)	26/37 and 27/38	anthranilate-CoA-ACP transferase and anthranilate-CoA ligase
ORF8 (RauG)	494	Acel_2081, *Acidothermus cellulolyticus* 11B (YP_873839)	37/56	efflux protein (MFS transporter)
ORF9 (RauH)	198	SSEG_02507, *Streptomyces sviceus* ATCC 29083 (YP_002207438)	50/59	isopentenyl diphosphate (IPP) δ-isomerase
ORF10 (RauI)	316	Noca_3084, *Nocardioides* sp. JS614 (YP_924273)	37/50	polyprenyl synthase (farnesyl synthase)
ORF11	425	no significant homologue was found	–	unknown



Scheme 2. Organization of the aurachin biosynthesis genes. Organization of *rau* genes of *R. erythropolis* JCM 6825 (top) and *aua* genes of *S. aurantiaca* Sg a15 (bottom). Large horizontal arrows indicate the location and direction of transcription of each predicted ORF. The small vertical arrow identifies the location of the insertion of the transposable element of pTNR. Deduced proteins that showed sequence similarities between *rau* and *aua* are shaded in gray and their sequence identities are indicated. The deduced protein of *rauF* also showed sequence similarity with that of *auaEII* (27% aa identity).

Gene inactivation of *rau* genes in strain JCM 6824

Of the 11 genes, the deleted mutants—ORF1, *rauA* (ORF2), *rauG* (ORF8), and ORF11—were constructed by the markerless gene disruption method with the *sucB* gene disruption system (for details see the Experimental Section). The mutant strains, designated strains M01, M02, M08, and M11, respectively, were grown in liquid culture medium, and the ethyl acetate extracts of the culture supernatants were subjected to HPLC analysis and growth inhibition bioassays with *A. atrocyaneus* as the indicator strain (Figure 1). Growth inhibition zones were detected with strains M01, M08, and M11, and production of aurachin RE in these strains was also confirmed by HPLC (retention time 10.6 min). These results clearly indicated that the three corresponding genes are not essential for the production of aurachin RE.

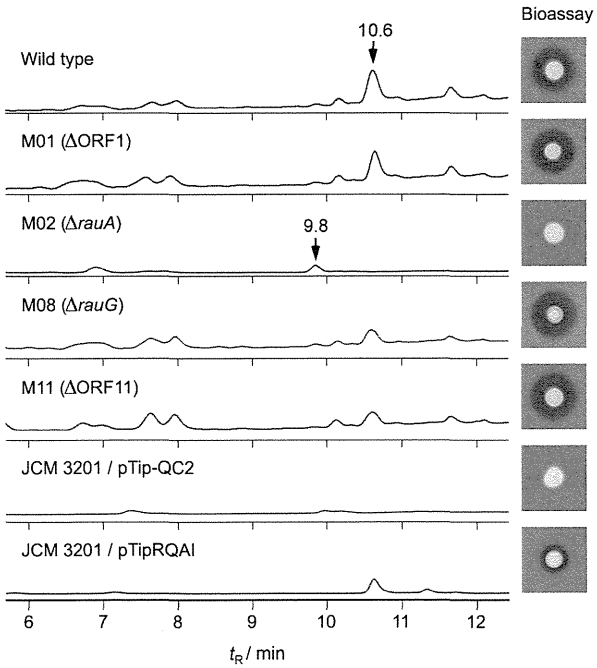


Figure 1. HPLC detection of aurachin RE and bioassay of wild-type JCM 6824 and modified strains. The culture supernatant of each strain was subjected to HPLC analysis, and aurachin RE was detected at the retention time of 10.6 min. A bioassay of each sample was also performed by using an *Arthrobacter* strain as the indicator strain (right).

Although the growth-inhibition zone and aurachin RE production could not be detected in M02, a possible intermediate at a retention time of 9.8 min, showing an UV/Vis absorption spectrum similar to that of aurachin RE, was observed by HPLC-DAD analysis. The accumulated possible intermediate from strain M02 was purified and used for the subsequent enzymatic study (see below).

These results clearly demonstrated that *rauA*-encoded cytochrome P450 was indispensable for the biosynthesis of aurachin RE. In addition to the four genes discussed above, the other *rau* genes were inactivated in a similar manner (Tables S1 and S2 in the Supporting Information). All the mutant strains lost the ability to produce the antibiotics; this clearly demonstrates that these genes are involved in, and are indispensable for, the production of aurachin RE.

rauA gene complementation in the mutant strain

To investigate *rauA* gene function, a gene complementation test was carried out in the mutant strain. *rauA* was PCR-amplified and ligated to the *Rhodococcus–E. coli* shuttle vector pK4.[10] The resulting plasmid was introduced into M02 cells by electroporation. The recombinant gene-complemented strain, C02, restored the growth inhibition activity against the indicator strain. Although it was not a complete recovery, HPLC-DAD analysis of the culture supernatant of the strain demonstrated that it had regained the capability for aurachin RE production (Figure S1). These results confirmed that *rauA* is functionally active and plays an essential role in the production of aurachin RE.

Heterologous expression of *rau* genes

In order to evaluate *rau* gene function in the heterologous host, *rauA–I* genes were ligated into the thiostrepton-inducible expression vector pTip-QC2,[11] and the resultant plasmid,

pTipRQAI, was introduced into the type strain of *R. erythropolis* (JCM 3201) by electroporation. The ethyl acetate extract of the culture supernatant of the recombinant was subjected to HPLC analysis and an antibacterial activity test. Under uninduced conditions, antibacterial activity was detected and product accumulation was demonstrated by HPLC analysis, at the retention time of 10.6 min (Figure 1). These results indicated that aurachin RE was successfully produced in the recombinant strain, probably as a result of basal level transcription of *rau* genes in the vector. However, the recombinant cells exhibited markedly diminished growth and weaker growth inhibition zone than the wild-type strain. Moreover, almost no growth was observed under thiostrepton-induced conditions, which was possibly due to the potent antibiotic activity of the products. These results again confirmed that the *rau* gene cluster was sufficient for the production of aurachin RE in *Rhodococcus* strains.

Identification of compound 2, a biosynthetic aurachin RE intermediate

As described above, accumulation of a possible aurachin RE intermediate was observed in culture media of the *rauA* mutant (strain M02). The mutant did not show growth inhibition activity, and the deduced amino acid sequence of RauA showed moderate identity (≈34%) with some cytochrome P450 monooxygenases in the databases. The heme-attaching cysteine residue, crucial for cytochrome P450 monooxygenases, was found to be conserved in the RauA sequence. These observations indicated that RauA is most likely a cytochrome P450 monooxygenase that plays an important role in the production of mature antibiotics. We therefore hypothesized that the accumulated intermediate found in M02 was a specific substrate for the P450 RauA. The intermediate—compound **2**—was isolated and purified from the culture media of strain M02. Its molecular formula was deduced to be $C_{25}H_{33}NO_2$ (M_W=379) by HRMS {*m/z* 362.2478 $[M+H-H_2O]^+$ (calculated exact mass for $C_{25}H_{32}NO$: 362.2478) and *m/z* 378.2441 $[M-H]^-$ (calculated exact mass for $C_{25}H_{32}NO_2$: 378.2439)}. This formula is consistent with aurachin RE lacking one oxygen atom.

The structure of compound **2** was elucidated by extensive NMR analysis; the ^1H and ^{13}C NMR chemical shifts are shown in Table 2. The ^1H NMR spectrum and HSQC analysis indicated the presence of 15 methyl protons, eight methylene protons, and eight methine protons, the last of which included three olefinic protons and four aromatic protons. The ^{13}C NMR spectrum indicated the presence of 25 carbon atoms, which were assigned to five methyl, four methylene, and eight methine systems, as well as eight carbon atoms without attached protons, by DEPT spectroscopy. DQF COSY and HMBC experiments confirmed the partial structures of compound **2** (COSY and HMBC correlations are illustrated in Figure S2). The whole structure was determined as shown in Scheme 1.

The structure of compound **2** was closely related to that of aurachin RE, with the sole difference between the two compounds being observed at the N atom in the quinolone ring. Aurachin RE has a hydroxy group on the N atom, whereas the intermediate **2** does not. The structure of **2**, which lacks one oxygen atom relative to that of aurachin RE, appeared to be a suitable substrate of the P450 RauA in the biosynthetic pathway.

Table 2. NMR spectral data for biosynthetic intermediate **2** and for aurachin RE (**1**).

	2[a]		**1**[b]	
	δ_C	δ_H (mult., *J* [Hz])	δ_C	δ_H (mult., *J* [Hz])
1		11.4 (s) (–N*H*)		
2	146.8		147.8	
3	118.7		117.9	
4	175.8		173.1	
4a	124.0		123.6	
5	125.7	8.01 (d, 7.9)	125.5	8.11 (dd, 1.4, 6.9)
6	122.9	7.21 (dd, 7.4, 7.5)	122.9	7.29 (t, 7.3)
7	131.4	7.53 (dd, 7.5, 7.6)	131.6	7.64 (dd, 1.4, 6.9)
8	118.0	7.43 (d, 8.2)	114.7	7.77 (d, 8.2)
8a	139.6		139.7	
9	18.1	2.31 (s)	14.7	2.39 (s)
1′	23.8	3.19 (d, 6.5)	24.2	3.29 (d, 6.8)
2′	123.4	4.96 (m)	123.1	4.98 (m)
3′	134.3		134.3	
4′	39.7[c]	1.88 (t, 7.2)	39.3	1.90 (m)
5′	26.7	1.96 (td, 7.0, 7,2)	26.4	1.99 (m)
6′	126.5	5.00 (m)	126.1	5.02 (m)
7′	132.4		132.1	
8′	48.6	1.84 (dd, 6.7, 13.1)	48.3	1.88 (m)
		2.03 (dd, 6.7, 13.1)		2.06 (m)
9′	66.5	4.18 (m)	66.1	4.21 (q, 7.3)
9′		4.26 (d, 4.6) (–O*H*)		
10′	130.3	4.98 (m)	130.0	5.00 (m)
11′	131.5		131.1	
12′	26.0	1.56 (s)	25.6	1.59 (s)
13′	16.5	1.69 (s)	16.2	1.72 (s)
14′	17.0	1.50 (s)	16.6	1.53 (s)
15′	18.5	1.49 (s)	18.1	1.52 (s)

[a] Measured in [D_6]DMSO. [b] The listed NMR data were reported previously by W.K. et al.[5] [c] The ^{13}C chemical shift value was determined from the DEPT 135 spectrum.

Functional analysis of RauA

To study the enzyme activity in vivo, the *rauA* gene was ligated into the pNit-QC2 vector to give pNit-rauA, and introduced into the *R. erythropolis* host strain (JCM 3201). A resting cell assay of the *Rhodococcus* recombinant (harboring pNit-rauA) was performed with the intermediate **2** as the substrate. HPLC-DAD analysis demonstrated the disappearance of **2** (t_R= 9.8 min) and the appearance of a single peak at a retention time of 10.6 min (data not shown). This peak showed the same retention time and UV–visible absorption spectrum as aurachin RE. In addition, the ethyl acetate extract of the culture supernatant of the recombinant showed antibiotic activity, whereas that of the blank vector control did not. These results strongly indicated that RauA converts the intermediate **2** into aurachin RE.

In order to elucidate the function of RauA, an in vitro enzyme assay was also performed. The RauA enzyme produced in *E. coli* was purified, and the enzyme assay was performed as described in the Experimental Section. HPLC-DAD analysis

clearly showed that aurachin RE was produced and the intermediate **2** was significantly consumed (Figure 2); this suggests that RauA catalyzes the hydroxylation of the N atom in the quinolone ring to complete the biosynthesis of aurachin RE and that the enzyme is responsible for the conversion of an inactive precursor into a mature antibiotic. The specific activity for the N-hydroxylation was calculated to be 2.9 ± 0.26 mol min^{-1} per mol of RauA. This value is comparable with those reported for other bacterial and mammalian cytochrome P450s that metabolize highly hydrophobic compounds.[12]

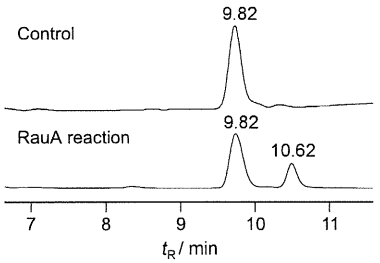


Figure 2. Transformation of precursor **2** into aurachin RE by purified P450 RauA. Aurachin RE and its precursor were detected by HPLC analysis at the retention times of 10.6 and 9.8 min, respectively. Control reaction lacks the RauA protein.

Discussion

The biosynthesis genes of aurachin RE were successfully isolated from the strain JCM 6824 with the aid of random transposon mutagenesis. The gene region contained 11 putative genes, designated the *rau* gene cluster. With respect to the aurachin biosynthetic system, the *aua* gene cluster had previously been isolated from the Gram-negative strain *S. aurantiaca* Sg a15, which produces aurachin C and its derivatives.[8] On comparison of the gene clusters, the deduced amino acid sequences of RauB, RauD, and RauF from *Rhodococcus* were found to show some similarity with those of AuaA (farnesyltransferase), AuaC (polyketide synthase, PKS), and AuaE/AuaEII (anthranilate-CoA-ACP transferase/anthranilate-CoA ligase), respectively, from *Stigmatella* (Table 1 and Scheme 2). Recently, the molecular function of AuaA prenyltransferase (membrane-bound farnesyltransferase) from Sg a15 was characterized.[13] Putative RauB prenyltransferase from JCM 6824 showed significant sequence similarity only with AuaA and, like AuaA, is also predicted to be a membrane protein with nine transmembrane helices.[14] In addition, *rauB* was indispensable for the production of aurachin RE. These results strongly suggested that RauB acts as the farnesyltransferase in the biosynthetic pathway. With respect to PKSs, only the β-ketoacyl (ACP) synthase II proteins (RauD and AuaC) showed significant similarity. The putative acyl carrier protein (ACP) and the second β-ketoacyl ACP synthase II protein of *Rhodococcus* (RauC and RauE) did not show sequence similarity with the *aua* gene products of *Stigmatella*. The putative RauF anthranilate-CoA ligase showed sequence similarity with a large number of benzoate-CoA ligase family proteins, especially with AuaE and AuaEII of strain Sg a15. Pistorius et al. have recently clearly demonstrated that both AuaE and AuaEII are involved in aurachin production.[15] These RauCDEF proteins would be expected to produce a quinoline ring from anthranilic acid and acetate, as proposed in the case of *Stigmatella*.[8,15]

In addition to these enzymes (or this enzyme complex), six genes were found in the *rau* gene cluster of *Rhodococcus* wherein the deduced proteins did not show similarity with any *aua* gene product of *Stigmatella*. The estimated amino acid sequence of ORF1 correspond to that of a TetR-type transcriptional regulator, which has been observed in many antibiotic biosynthesis gene clusters, and works as a repressor (down-regulator). Gene inactivation in the ORF1 mutant (strain M01), however, resulted in no increase in aurachin RE production ability, thus indicating that ORF1 is unrelated to aurachin biosynthesis.

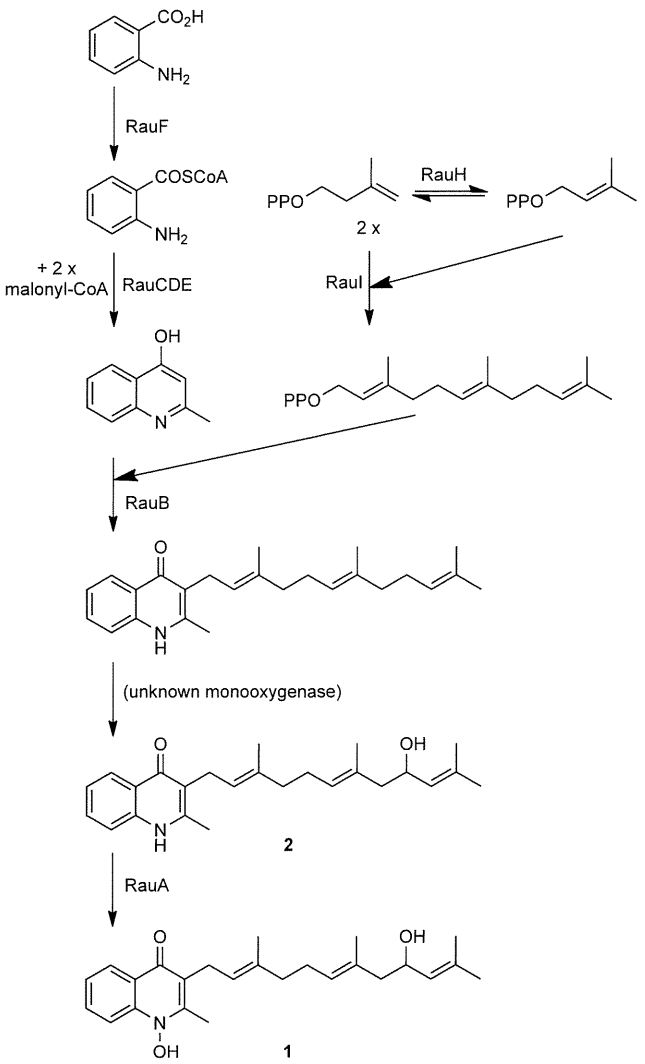
The RauA protein was found to be a cytochrome P450 monooxygenase that catalyzes N atom hydroxylation of the intermediate compound to produce aurachin RE. Because the intermediate **2** has no antibiotic activity, RauA plays a very important role in the biosynthetic pathway by endowing the molecule with antibiotic properties. Surprisingly, N-hydroxylation was not catalyzed by cytochrome P450 monooxygenase in the case of aurachin C in *S. aurantiaca* Sg a15. Recently, Pistorius et al. also demonstrated that N-hydroxylation was instead catalyzed by AuaF, a Rieske [2Fe–2S] oxygenase (Scheme 2).[16] The authors also reported that five cytochrome P450 monooxygenase genes found in the draft genome sequence of Sg a15 could not be involved in aurachin production because none of the gene mutants was deficient with respect to the production of hydroxylated compounds. In the case of JCM 6824, P450 RauA is a unique protein that plays a crucial role in oxygenation.

The RauG protein showed some similarity with the MFS transporter. The predicted amino acid sequence of RauG had 14 putative transmembrane regions and has been shown to be a member of the drug efflux subfamily of MFS, the function of which depends on proton motive force.[17] Many drug efflux pump proteins (or the corresponding genes) have been found in microorganisms, and some proteins in this superfamily have been found to confer antibiotic resistance on the producing species and other susceptible microorganisms.[18] In the case of *rauG*, the mutant strain (strain M08) retained aurachin RE productivity, and these products were isolated only from the culture supernatant (data not shown). Additionally, *rauG* did not confer antibiotic resistance on the host *Rhodococcus* strain through the action of the constitutive expression vector (data not shown). The function of RauG therefore remains unknown.

The RauH and RauI proteins showed strong similarity with proteins of the isopentenyl diphosphate isomerase and polyprenyl synthase families, respectively. These two enzymes are involved in the synthesis of farnesyl pyrophosphate to provide the prenyl chain moiety of aurachin RE. The estimated amino acid sequence of ORF11 was predicted to be that of a membrane protein, due to its putative nine transmembrane helices. However, it showed no similarity to any known protein in the

databases. Because the mutant strain (M11) had no effect on the production of aurachin RE, the function of the ORF11 protein also remains unknown.

A proposed aurachin RE biosynthetic pathway in *Rhodococcus*, based on the results of this study and previous reports relating to *Stigmatella*, is shown in Scheme 3. Of these 11 genes,



Scheme 3. Proposed biosynthetic pathway for aurachin RE (**1**) and corresponding gene products in *R. erythropolis* JCM 6824.

the functional roles of the gene products (ORF1 and RauA, -B, -C, -D, -E, -F, -H, and -I) of nine genes were elucidated or estimated. On the other hand, some important genes seemed to be missing in the gene cluster. Firstly, there was no evidence of any gene that could be involved in self-resistance. Aurachin RE shows strong antibiotic activity against Gram-positive bacteria and is also active against *Rhodococcus* species. Although the antibiotic mechanism and resistance mechanism are still unknown, our results indicate that the self-resistance gene must lie somewhere outside of the *rau* gene cluster. Secondly, no electron-transfer component genes for P450 RauA were found. A cytochrome P450 monooxygenase generally works in conjunction with ferredoxin and ferredoxin reductase. Therefore, the electron-transfer components that could couple with P450 RauA must be encoded in a different locus on the genome. Thirdly, the genes for hydroxylation of the prenyl chain at C9′ also seemed to be missing.

In the heterologous expression experiment, the *rau* genes were sufficient to confer production ability for aurachin RE on the host strain *R. erythropolis* JCM 3201. This suggested that the strain JCM 3201 provided suitable proteins to compensate the electron-transfer component for the RauA. Also, the host strain might possess hydroxylating enzymes capable of hydroxylating C9′ of the prenyl chain moiety of aurachin. Only minor amounts of aurachin RE were produced by the heterologous host strain with use of the pTip-QC2 expression vector under uninduced conditions. When the constitutive expression vector pNit-QC2 was used (resulting in pNitRQAI), no transformant was obtained in repeated trials. This might have been due to the absence of an antibiotic resistance system. Indeed, the natural resistance level of the host strain to aurachin RE might be very low (MIC = 0.1 $\mu g\,mL^{-1}$).[5] Hence, the recombinant containing the *rau* gene cluster might not have been able to produce large amounts of the antibiotic, and in some cases, might not have been able to survive, due to the lethal effects of the produced antibiotic. This observation again confirmed that no gene in the *rau* cluster was involved in antibiotic resistance.

As discussed above, certain relationships between the *rau* genes of Gram-positive *Rhodococcus* and the *aua* genes of Gram-negative *Stigmatella* were confirmed in this study. However, many more dissimilarities between these production systems were also found:

1) Aurachin RE of *Rhodococcus* was recovered from culture medium, whereas the products of *Stigmatella* have mostly been isolated from cell mass.[6] Because aurachin antibiotics are particularly active against Gram-positive bacteria, *Rhodococcus* might have to expel it outside its cells.
2) *Rhodococcus* produces only C-type aurachins (farnesyl chain on C-3), whereas *Stigmatella* produces both C-type and A-type (farnesyl chain on C-4) aurachins.
3) Aurachin RE had a hydroxy group on the farnesyl chain, whereas none of the aurachins from *Stigmatella* had a hydroxy group on that moiety.
4) The organization of the *rau* gene cluster is mostly different from that of the *aua* gene cluster. Of the 11 genes, only three showed sequence similarity with *aua* genes. Furthermore, the *rau* gene cluster contained *rauH* and *rauI* genes for the production of the farnesyl chain, and no corresponding genes have been found in any locus of the *aua* gene cluster.
5) Most interestingly, the role played by the cytochrome P450 monooxygenase gene in the cluster in *Rhodococcus* is instead played by a Rieske [2Fe–2S] oxygenase in *Stigmatella*.

In view of these differences, it could not be conclusively established whether or not these gene clusters have a common

ancestral origin; however, the evolutionary relationship between the two appears to be distant. The fact that the two clusters produce similar secondary metabolites might be a result of convergent evolution rather than shared ancestry. Further studies will be required in order to achieve better overall understanding of the aurachin RE antibiotic, with particular emphasis on its self-resistant system and hydroxy group modification.

Conclusions

To date, two antibiotic peptides[4] and two aurachins[5,19] have been isolated in the genus *Rhodococcus*; however, no details of their biosynthesis genes had been put forth until now. We have cloned an aurachin RE biosynthesis gene cluster from *R. erythropolis* JCM 6824. This is the first example of the identification of an antibiotic producing gene from the genus *Rhodococcus*, since the identification of this group of bacteria as potent antibiotic producers. To the best of our knowledge, this study is also the first to demonstrate heterologous antibiotic production in the genus *Rhodococcus*. Our study confirmed that our expression vectors are very useful in *Rhodococcus* and also demonstrated that *Rhodococcus* is a good expression host microorganism for bioproduction, even for secondary metabolite compounds. As we have reported previously,[3] the genus *Rhodococcus* contains several antibiotic producers. We plan to use our transposon and expression system for the isolation of new antibiotics and biosynthesis genes from this genus.

In this study, we identified a new functional P450 monooxygenase, which catalyzes N-hydroxylation in the quinolone ring skeleton of the aurachin precursor. To the best of our knowledge, this is the first study to identify a P450 enzyme that introduces a hydroxy group onto the N atom in the quinolone ring. This is the most important enzyme for the production of the antibiotic because the introduction of the hydroxy group finalizes the production process and also endows the compound with antibiotic properties. This unique function of P450 might therefore be useful for the development of new antibiotic products with quinolone and/or similar ring compounds as precursors.

Experimental Section

Bacterial strains, plasmids, and culture conditions: The bacterial strains and plasmids used in this study are listed in Table S1. *Rhodococcus* and *Arthrobacter* strains were grown in lysogeny broth (LB) medium at 28 °C, and *E. coli* strains were grown in LB medium at 37 °C. When antibiotics were required, they were used at final concentrations of 100 $\mu g\,mL^{-1}$ for ampicillin, 34 $\mu g\,mL^{-1}$ for chloramphenicol, and 200 $\mu g\,mL^{-1}$ for kanamycin. The growth inhibition activity of *Rhodococcus* strains was tested on LB soft-agar medium (soft-agar overlay assay) with *Arthrobacter atrocyaneus* as the indicator strain.[3] W-minimal medium[20] supplemented with succinate (0.2%, *w/v*), sucrose (0.2%, *w/v*), and casamino acids (0.2%, *w/v*) was also used to isolate the antibiotic aurachin RE and its derivatives from *Rhodococcus* strains.

Extraction, purification, and identification of aurachin RE and derivatives: To recover aurachin RE and its derivatives from liquid culture media, the fermentation broth (6.0 L) was centrifuged, and any residual cells in the supernatant were removed by membrane filtration (0.2 μm pore size). The filtered supernatant was applied to a Diaion HP-20 and C18 cartridge column (SepPak Vac 35 cc, Waters, Milford, MA), and the column was sequentially washed with EtOH (20%, 100 mL) and EtOH (50%, 30 mL). The sample was eluted with EtOH (20 mL), followed by ethyl acetate extraction, concentrated to dryness, analyzed, and purified by HPLC. Analytical reversed-phase HPLC experiments were performed with a C18 column (TOSOH TSKgel ODS-80Ts, 4.6 mm i.d.×15 cm) in a Hitachi Elite LaChrom system with diode array detection (HPLC-DAD; monitor wavelength, 220–600 nm). The following H_2O/MeOH mobile phase was used with a flow rate of 1 $mL\,min^{-1}$: 68–78% MeOH (0–7 min), 78–90% MeOH (7–7.5 min), and 90% MeOH (7.5–12 min). Under these conditions, aurachin RE (**1**) and the biosynthetic intermediate **2**, which was isolated from the *rauA* mutant strain M02, were eluted at retention times of 10.6 min and 9.8 min, respectively (Figure S3). Preparative HPLC of the intermediate was performed with a C18 column (TOSOH TSKgel ODS-80Ts, 20 mm i.d.×25 cm) and an acetonitrile (48%) mobile phase at a flow rate of 8 $mL\,min^{-1}$. The intermediate compound **2** was eluted at a retention time of 44.1 min. The structures of these compounds were assigned on the basis of their mass and NMR spectral data. High-resolution mass spectra were collected with a Triple TOF 5600 System (AB SCIEX, Tokyo, Japan) operating in the ESI^+ and ESI^- modes, and NMR spectra were recorded with an ECA-600 NMR spectrometer (600 MHz, JEOL, Tokyo, Japan).

Isolation of *rau* gene mutants: Random transposon mutagenesis and subsequent nucleotide sequencing were carried out by use of a pTNR plasmid as described previously.[9] Targeted gene disruption of the aurachin RE biosynthesis genes (*rau*) was carried out by homologous recombination with the *sucB* counter-selection system.[21] The primers used in this study are listed in Table S2. To disrupt *rauA* (ORF2) on the chromosome, a gene disruption plasmid (suicide vector) was constructed as follows: a 1.4 kb region containing a partial 5′-terminal sequence of *rauA* and a 1.3 kb region containing a partial 3′-terminal sequence of *rauA* were PCR-amplified with use of primers that contained a restriction enzyme recognition sequence. Neither of these amplified fragments contained the central part (0.9 kb) of the *rauA* sequence. These two fragments were digested with EcoRI-BamHI and BamHI-PstI, respectively, followed by ligation with the pK18mobsacB plasmid at the EcoRI-PstI site. The resulting plasmid, pK18M02, was introduced into wild-type JCM 6824 cells by electroporation. Transformants were selected on LB agar plates containing kanamycin (200 $\mu g\,mL^{-1}$) and subjected to PCR screening to identify genuine single-crossover mutants, with use of aphII-UR and ORF2-Single-C primers (Table S2). With this primer set, 2.8 kb or 3.8 kb amplified fragments were obtained from genuine mutants (homologous recombinants). Single-crossover mutants were then grown in liquid LB without kanamycin to generate double-crossover mutants. Transformants were selected on LB agar plates containing sucrose (10%, *w/v*) and subjected to PCR screening to identify deleted gene double-crossover mutants, with use of ORF2-DCC-F and ORF2-DCC-R primers (Table S2). With this primer set, a 1.7 kb amplified fragment was obtained from the revertant double-crossover clone (corresponding to the wild type), a 0.8 kb amplified fragment was obtained from the deleted gene double-crossover clone, and both fragments were obtained from clones that remained in the single-crossover state. This method enables markerless gene deletion, and in this case it only removed a 0.9 kb internal *rauA* nucleotide region from the chromosome. The resultant *rauA* mutant, designated as strain M02, was further subjected to Southern hybridiza-

tion to confirm the gene deletion with use of the *rauA* gene probe. All of the other ten *rau*-deleted mutants (strains M01 and M03–M11) were obtained in the same manner as described for *rauA*. The deleted regions of the corresponding *rau* genes are illustrated in Figure S4.

Functional analysis for P450 RauA

1) In vivo analysis of RauA in Rhodococcus *cells*: PCR amplification of *rauA* was carried out with JCM 6824 genomic DNA and the oligonucleotide primers rauA-F-NdeI and rauA-R-HindIII (Table S2). The amplicon was digested and ligated into the *Rhodococcus* expression vector pNit-QC2[11] to generate pNit-rauA, and the construct was introduced into the expression host; that is, *R. erythropolis* strain M1218. A resting cell assay was performed as follows: the *rauA* recombinant cells grown on LB medium at 28 °C for 10 h were washed twice with W-minimal medium and were adjusted to an optical density at 600 nm (OD_{600}) in the medium (2.5 mL). The cells were incubated with the biosynthetic intermediate **2** (10 μg mL^{-1}) at 28 °C for 2 h with gentle shaking. After incubation, ethyl acetate (2.5 mL) was added to extract the reaction products. The organic extract was then concentrated to dryness under vacuum, and the residue was reconstituted with ethanol (100 μL) in preparation for HPLC or antibiotic analysis.

2) In vitro analysis of RauA: The *rauA* gene was PCR-amplified with the primers rauA-F-NdeI and rauA-XhoI-His-R and ligated into a pET26 vector (Novagen). The recombinant P450 RauA, containing a hexahistidine tag at the C terminus, was overexpressed in *E. coli* BL21-CodonPlus(DE3)-RIL cells. P450 RauA expression was induced with isopropyl β-D-thiogalactoside (IPTG, 0.1 mM) in LB supplemented with $FeSO_4$ and 5-aminolevulinic acid (heme precursor), by previously described methods.[22] The cells were harvested and resuspended in a buffer (buffer A) consisting of Tris·HCl (pH 7.5, 50 mM) and glycerol (10%). The cells were lysed by sonication, and the homogenate was clarified by centrifugation. The supernatant was applied onto an Ni-affinity column (His-Select, Sigma–Aldrich) pre-equilibrated with buffer A. The His-tagged P450 RauA was eluted with a linear gradient of imidazole in buffer A (0–400 mM). The red fractions were collected, dialyzed against a buffer (buffer B) consisting of Tris·HCl (pH 7.5, 20 mM), NaCl (100 mM), dithiothreitol (DTT, 1 mM), and glycerol (10%), and subsequently loaded onto a DEAE Sephacel Fast Flow column (GE Healthcare) pre-equilibrated with buffer B. The bound proteins were eluted with a linear gradient of NaCl in buffer B (100–500 mM). The individual fractions were analyzed by SDS-PAGE. The concentration of P450 RauA was determined by reduced carbon monoxide difference spectral analysis,[23] with a JASCO V-630 biospectrophotometer. The enzymatic reaction catalyzed by the recombinant RauA was examined by means of reconstitution experiments. The reaction mixture contained RauA (2.5 μM), substrate (biosynthetic intermediate **2**, 2.5 μM), spinach ferredoxin (Fdx, Sigma–Aldrich, 100 μg mL^{-1}), spinach ferredoxin reductase (FdxR, Sigma–Aldrich, 0.1 U mL^{-1}), D-glucose dehydrogenase (Toyobo, 3 U mL^{-1}), NADPH (2 mM), and D-glucose (60 mM) in a total volume of 200 μL of reaction buffer (Tris·HCl, pH 7.5, 50 mM). The spinach Fdx and FdxR are commonly used as redox partners for the P450 assay.[24] After pre-incubation at 30 °C for 2 min, the reaction was initiated by the addition of NADPH, followed by incubation at 30 °C for 5 min. The reaction was terminated by extraction once with ethyl acetate (800 μL). The resulting organic extract was dried and redissolved in methanol (100 μL). The methanol solution was analyzed by HPLC by the same procedure as described above. The molar quantities of aurachin RE and the biosynthetic intermediate **2** were estimated from the previously reported molar absorption coefficient for aurachin D at one of the major absorption peaks (334 nm).[6]

Accession numbers: The nucleotide sequence determined in this study has been deposited in the DDBJ, EMBL, and GenBank databases under accession no. AB694012.

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Keywords: antibiotics · aurachin · biosynthesis · gene clusters · P450 · *Rhodococcus*

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Supporting Information

Cloning and Heterologous Expression of the Aurachin RE Biosynthesis Gene Cluster Afford a New Cytochrome P450 for Quinoline N-Hydroxylation

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