3．維持管理基準の確認

主に屋内で捕獲されるチカイエカに以下の基準を確認する。

快適基準：以下のすべてに該当すること。

①トラップによる捕獲指数が１未満。

②1個のトラップに捕獲される数が１日当たり１匹以下。

③屋内に生きたチカイエカが目撃されない。

警戒基準：以下のすべてに該当すること。

①トラップによる捕獲指数が１以上３未満。

②1個のトラップに捕獲される数が１日当たり２匹以下。

③トラップには捕獲されないが。屋内に生きたチカイエカが僅かに目撃される。

措置基準：以下のいずれか一つ以上に該当すること。

①トラップによるチカイエカ指数が３以上。

②1個のトラップに捕獲される数は１日当たり３匹以上。

③トラップには捕獲されないが、屋内で吸血される。

注：捕獲指数は１日、１トラップ当たりの捕獲数として表すこと。

4．作業計画

必要な措置に応じて、人員、使用薬剤・資材、機器を手配し、実施スケジュールなど作業計画を策定する。

5．防除作業

5－1　環境的な対策

（1）幼虫対策

①　マンホールがある水槽では、水槽内部と隣接の水槽との間に貫通している隙間や連通管に防虫ネットを設置する。水槽内部は有毒ガスが発生している恐れがあるので、作業は工事業者に依頼する。

②　水槽はできるだけ頻繁に水抜きなど清掃を行う。

（2）成虫対策

①　窓などに対して網戸を設置する。

②　換気口、ドアーの隙間をチェックし、不備があれば補修する。

5－2　薬剤を用いた対策

（1）事前通知

薬剤を処理する場合は、少なくとも３日前までに使用薬剤名、実施場所、においの程度、化学物質などに対する過敏者への注意などを記載した事前通知書を作成し、

実施３日後まで当該場所入り口に掲示しておく。空間噴霧を行った場所で、人の出入りがある場所では、処理後、窓などを開放し、少なくとも３時間は立入禁止にする。

（２）幼虫対策

①　発生水域の容量や実際の水量を測定する。

②　マンホールがない水槽では、薬剤投入のため床面に小さな穴（ピット）を設ける。

③　有機リン剤やIGR剤を用法・用量、使用上の注意を守って水域に処理する。

（３）成虫対策

①　発生のある又は発生が予想される水槽内及び飛翔区域にULV処理等により空間噴霧する。

②　水槽内には樹脂蒸散剤を吊す。

５－３　効果判定

防除終了後、事前調査と同じ方法で効果判定を行う。

５－４　再作業

効果判定によって警戒または措置基準を超えている場合には、再度調査を行って問題点を明らかにし、再作業を行う。

５－５　報告書の提出

対策の結果を詳細に文書で関係者に報告する。管理上の問題点などがあれば指摘をする。

５－６　緊急対応整備

防除作業及び機械器具設備の維持管理に係る苦情及び緊急の連絡に対して、迅速に対応できる体制を整備しておく。

研究成果の刊行に関する一覧表

雑誌

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# Dry ice-trap collection of mosquitoes flying near a tree canopy in Nagasaki, Japan, with special reference to *Aedes albopictus* (Skuse) and *Culex pipiens pallens* Coquillett (Diptera: Culicidae)

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**Abstract:** Flying mosquitoes were collected at a pair of collection sites at different heights from the ground by a suction trap enhanced with 1 kg of dry ice in summer in 2001 and 2002, on the campus of Nagasaki University, School of Medicine, and on a small desert island in Nagasaki, Japan. One collection site was near a tree canopy (6–12 m above the ground) and the other one at about 1 m above the ground. At each collection site, the trap was operated for 24 h to collect both nocturnal and diurnal species. The following 9 species were encountered; *Aedes albopictus* (Skuse), *Ochlerotatus nipponicus* LaCasse et Yamaguti, *Culex pipiens pallens* Coquillett, *Armigeres subalbatus* (Coquillett), *Cx. tritaeniorhynchus* Giles, *Cx. halifaxi* Theobald, *Cx. bitaeniorhynchus* Giles, *Orthopodomyia anopheloides* (Giles), *Tripteroides bambusa* (Yamada). The proportion of mosquitoes collected near the tree canopy was calculated for 4 dominant species; 3.9 (females) and 1.2% (males) for *Ae. albopictus*, 64.5% for females of *Cx. pipiens pallens*, 19% for females of *Ar. subalbatus* and 26.7% for females of *Cx. tritaeniorhynchus*. The vertical distribution of flying mosquitoes in relation to the location of their vertebrate host is discussed.

Key words: vertical distribution, mosquitoes, *Aedes albopictus*, *Culex pipiens pallens*, dry ice trap

## INTRODUCTION

Vertical distributions of flying mosquitoes have been examined mainly in West Africa (Snow, 1975, 1979, 1982; Gilles and Wilkes, 1976; Haddow et al., 1961; Corbet, 1961a,b; Snow and Wilkes, 1977; Gillies, 1988; Clements, 1999), and no information is available for Japanese mosquitoes. In some field studies, ornithophagous mosquitoes, such as *Culex weshei* in West Africa and *Cx. pipiens* and *Culiseta morsitans* in England, were caught at higher elevations than near the ground (Snow, 1975, 1982; Gillies and Wilkes, 1976; Service, 1971) suggesting the overlap of vertical distribution of feeding mosquitoes with that of their vertebrate host (Clements, 1999). Clarification of the place of feeding and the blood source of mosquitoes is essential to understand the transmission dynamics of mosquito-borne diseases, which include wild animals as well as humans in the transmission cycle, like West Nile Virus in USA (Bernard et al., 2000; Kulasekera et al., 2000).

*Aedes albopictus* (Skuse) is a dominant mosquito in Nagasaki city, Japan (Iriarte et al., 1991; Tsuda et al., 1994), and shows

a wide range of host animals including man and birds, although the feeding pattern largely depends on the availability of the host animals (Hawley, 1988). Sakakibara (1980) observed egg-laying activities of *Ae. albopictus* in Mie, Japan by using ovi-traps hung at different heights from the ground, and found that about 60 and 5% of eggs were laid in the ovi-trap on the ground and the highest position (6 m above the ground), respectively. *Culex pipiens pallens* Coquillett is the most common mosquito in human dwelling areas in Japan and is primarily an avian feeders, while they feed severely also on man and other mammals (Tanaka et al., 1979). Because of the clear difference in blood-feeding periodicity, host-seeking behavior and host preference between the two species, a different vertical distribution of flying mosquitoes is expected between them.

By using a dry ice trap, the present study was conducted to compare the vertical distribution of flying mosquitoes between *Ae. albopictus* and *Cx. pipiens pallens* in two study areas, an urban area and a small desert island where availability of host animals was different.

## Materials and Methods

*Study area*

Two areas were selected for this study; one was located at the northern edge of a small woods on the campus of Nagasaki University, School of Medicine, Nagasaki, Japan, and the other one was on a small desert island, Maejima, which was located about 40 km east of Nagasaki city, Japan (32°45′34.2″ N, 130°02′26.7″ E). The study area on the campus of Nagasaki University had a dense vegetation of trees, shrubs, and herbaceous plants, and was surrounded by human dwellings. The size of Maejima Island was about 400 m by 200 m (8.3 ha) and the distance from the opposite seashore to the island was about 500 m. Dense vegetation of evergreen trees, shrubs as well as under-growing grasses covered nearly the whole island. The available host animals on the campus of Nagasaki University, School of Medicine were men, birds, cats, rats and dogs, whereas on Maejima Island rats were rare and probably only birds were available as a blood source of mosquitoes because of the small size of the island and no human dwellings on the island.

*Mosquito collection*

Flying mosquitoes were collected at a pair of collection sites with different height from the ground by a suction trap with 1 kg of dry ice operated by batteries in August and September 2001, and in June and July 2002. The design of the suction trap was similar to the CDC-light trap (Service, 1993); made from a 14 cm length of 8.5 cm internal diameter acrylic tubing, and a 3.0-V motor with a three-bladed fan made of plastic was operated from four 1.5-V dry batteries. The dry ice was wrapped with paper and kept in a Styrofoam-box. A piece ofdryicealways remained in thebox after 24 h of collection. Five and two trees growing at the forest fringe were selected on the campus of Nagasaki University, School of Medicine and on Maejima Island, respectively. A rope was hung on a branch of the tree, and two traps were hung at different positions by using the rope; one trap hung near the tree canopy and the other one

Table 1. Height of collection site (m) from the ground examined in this study.

| Position | Campus of Nagasaki University | | | | | Maejima Island | |
|---|---|---|---|---|---|---|---|
| | Tree A | Tree B | Tree C | Tree D | Tree E* | Tree 1 | Tree 2 |
| High | 11.0 | 9.0 | 6.2 | 6.4 | 12.0 | 7.9 | 8.0 |
| Low | 1.0 | 0.5 | 0.7 | 1.0 | 1.0 | 1.0 | 1.0 |

*Middle position was used additionally at 6 m from the ground for Tree E.

near the ground. The height of each collection site from the ground examined in this study is shown in Table 1. The trap was operated 24 hr to collect both nocturnal and diurnal mosquito species.

## Results

The following 7 mosquito species were encountered on the campus of Nagasaki University, School of Medicine (Table 2); *Ae. albopictus*, *Cx. pipiens pallens*, *Armigeres subalbatus*, *Cx. tritaeniorhynchus*, *Tripteroides bambusa*, *Cx. halifaxi*, *Orthopodomyia anopheloides*. A total of 1,703 females of *Ae. albopictus* were collected from Trees A–D, and 3.9% of them were trapped near the tree canopy. The percentage of males of *Ae. albopictus* trapped near the tree canopy was only 1.2% (7/603) and significantly lower than that of females ($\chi^2=10.706$, $P=0.001$). While in *Cx. pipiens pallens*, the percentage of females trapped at near the tree canopy was 64.5% (394/611) and significantly higher ($\chi^2=1037.110$, $P<0.001$) than that of *Ae. albopictus*.

On Maejima Island the following 6 species were collected (Table 3) and *Ae. albopictus* was the most abundant; *Ae. albopict-*

Table 2. Results of dry ice trap collection at a pair of collection sites on the campus of Nagasaki University, School of Medicine, in August 27–September 14, 2001 and June 4–14, 2002, Nagasaki, Japan.

| Species | | Position | | | | | | | | | | % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Low | | | | | High | | | | | |
| | | Tree A (1 m) | Tree B (0.5 m) | Tree C (0.7 m) | Tree D (1 m) | Total | Tree A (11 m) | Tree B (9 m) | Tree C (6.2 m) | Tree D (6.24 m) | Total | High position |
| *Aedes albopictus* | ♀ | 304 | 193 | 704 | 436 | 1,637 | 14 | 3 | 27 | 22 | 66 | 3.9 |
| | ♂ | 78 | 85 | 254 | 179 | 596 | 4 | 1 | 1 | 1 | 7 | 1.2 |
| *Armigeres subalbatus* | ♀ | 1 | 1 | 4 | 11 | 17 | 0 | 0 | 1 | 3 | 4 | 19.0 |
| *Culex pipiens pallens* | ♀ | 4 | 31 | 78 | 104 | 217 | 8 | 74 | 182 | 130 | 394 | 64.5 |
| | ♂ | 0 | 7 | 0 | 2 | 9 | 0 | 0 | 0 | 0 | 0 | 0.0 |
| *Cx. tritaeniorhynchus* | ♀ | 1 | 2 | 2 | 6 | 11 | 1 | 0 | 2 | 1 | 4 | 26.7 |
| *Cx. halifaxi* | ♀ | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 2 | 66.7 |
| *Orthopodomyia anopheloides* | ♀ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 100.0 |
| *Tripteroides bambusa* | ♀ | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0.0 |
| | ♂ | 0 | 1 | 0 | 6 | 7 | 0 | 0 | 0 | 0 | 0 | 0.0 |
| Number of days examined | | 9 | 10 | 20 | 20 | | 9 | 10 | 20 | 20 | | |

Table 3. Results of dry ice trap collection at a pair of collection sites on a desert island, Maejima, in September 3–7, 2001, Nagasaki, Japan.

| Species | | Position | | | | | | % |
|---|---|---|---|---|---|---|---|---|
| | | Low | | | High | | | |
| | | Tree 1 (1 m) | Tree 2 (1 m) | Total | Tree 1 (7.9 m) | Tree 2 (8 m) | Total | High position |
| *Ae. albopictus* | ♀ | 25 | 60 | 85 | 2 | 4 | 6 | 6.6 |
| | ♂ | 0 | 2 | 2 | 0 | | 0 | 0 |
| *Ochlerotatus nipponicus* | ♀ | 1 | 1 | 2 | 0 | 2 | 2 | 50 |
| *Ar. subalbatus* | ♀ | 11 | 1 | 12 | 2 | 0 | 2 | 14.3 |
| *Cx. pipiens pallens* | ♀ | 0 | 0 | 0 | 1 | 0 | 1 | 100 |
| *Cx. tritaeniorhynchus* | ♀ | 10 | 0 | 10 | 2 | 1 | 3 | 23.1 |
| *Tr. bambusa* | ♀ | 3 | 0 | 3 | 0 | 0 | 0 | 0 |
| Number of days examined | | 5 | 5 | | 5 | 5 | | |

Table 4. Results of dry ice trap collection at 3 different positions for Tree E on the campus of Nagasaki University, School of Medicine, in July 3–5, 2002, Nagasaki, Japan.

| Species | | Position | | | Total |
|---|---|---|---|---|---|
| | | Low (1 m) | Middle (6 m) | High (12 m) | |
| *Ae. albopictus* | ♀ | 181 (98.4) | 2 (1.1) | 1 (0.5) | 184 (100) |
| | ♂ | 148 (99.3) | 1 (0.7) | 0 (0) | 149 (100) |
| *Ar. subalbatus* | ♀ | 1 (50.0) | 1 (50.0) | 0 (0) | 2 (100) |
| *Cx. pipiens pallens* | ♀ | 4 (4.7) | 72 (83.7) | 10 (11.6) | 86 (100) |
| *Cx. tritaeniorhynchus* | ♀ | 5 (62.5) | 2 (25.0) | 1 (12.5) | 8 (100) |
| *Cx. halifaxi* | ♀ | 0 (0) | 1 (100) | 0 (0) | 1 (100) |
| *Cx. bitaeniorhynchus* | ♀ | 0 (0) | 1 (100) | 0 (0) | 1 (100) |

The value in parentheses shows the percentage.

*us*, *Ochlerotatus nipponicus*, *Ar. subalbatus*, *Cx. pipiens pallens*, *Cx. tritaeniorhynchus*, and *Tr. bambusa*. The percentage of female *Ae. albopictus* trapped near the tree canopy was 6.6% (6/91). The difference in the percentage of *Ae. albopictus* trapped near the tree canopy was not significant between the campus of Nagasaki University and Maejima Island ($\chi^2=1.656$, $P=0.198$). The small number of *Cx. pipiens pallens* on Maejima Island was ascribed mainly to the scarcity of breeding sites on the island. There were no breeding sites on the island also for *Cx. tritaeniorhynchus*, however, 13 females were collected during the study. These females might immigrate from the mainland, since this species has good dispersal ability and can disperse >10 km (Wada et al., 1969).

On Tree E which was taller than Trees A–D on the campus of Nagasaki University, 3 dry ice traps were hung at 3 different positions; low (1 m), middle (6 m) and high (12 m). A total of 6 species were collected and a sufficient number of *Ae. albopictus* and *Cx. pipiens pallens* were collected for statistical analysis (Table 4). The composition of female mosquitoes trapped at the 3 different positions was significantly different between *Ae. albopictus* and *Cx. pipiens pallens* ($\chi^2=238.818$, $P<0.001$). More than 80% of *Cx. pipiens pallens* was trapped at the middle position and only 4.7% was trapped near the ground, while in *Ae. albopictus* a large part of females (98.4%) was trapped near the ground.

## Discussion

A clear difference in vertical distribution of flying mosquitoes was found between *Ae. albopictus* and *Cx. pipiens pallens* in this study which was similar to the contrast between *Ae. cantans* and *Cx. pipiens* observed in England (Service, 1971). *Culex pipiens pallens* are basically avian feeders (Tanaka et al., 1979) and more than 60% of females were collected near the tree canopy, while more than 90% of *Ae. albopictus*, which prefers to feed upon mammals (Hawley, 1988), was collected near the ground in our study. Although the proportion of *Ae. albopictus* trapped near the tree canopy was low, birds might be one of the host animals of *Ae. albopictus* in Nagasaki, Japan since this species showed high feeding activity even in night time (Higa et al., 2000) and high possibility to encounter birds during the night is expected for host-seeking females. Because *Ae. albopictus* is highly susceptible to West Nile Virus (Turell et al., 2001; Sardelis et al., 2002), it could be an important bridge vector of West Nile virus from wild birds to human.

There was no human dwelling on Maejima Island. Mammals, such as rats and rabbits, were rare and birds were the most abundant host animals of *Ae. albopictus* on the island. Therefore, the proportion of females trapped near the tree canopy was expected to be higher on Maejima Island

than that on the campus of Nagasaki University where cats, dogs, rats and human as well as birds were available for biting females. However, the percentage of *Ae. albopictus* females trapped near the tree canopy was not significantly different between the two study areas in this study. This result suggested that host-seeking behavior of *Ae. albopictus* was not affected by the availability of host animals.

The vertical distribution of *Cx. pipiens pallens* observed on Tree E as well as Trees A–D suggested the importance of a tree canopy for biting females in determining the place of feeding. The canopy of Tree E had a double layer, and the middle trap was hung at near the lower layer. Although the trap at the highest position was hung near the top layer, the layer was thin and often exposed to strong wind. These differences in environmental conditions of the tree canopy between the high and middle positions on Tree E might be the main reason for the highest proportion of collected females (>80%) at the middle position.

Our results suggested that not only the distribution of host animals but also other factors, such as flight ability, host-seeking behavior and microclimate conditions around the tree canopy, might determine the vertical distribution of flying mosquitoes.

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# YEAST-GENERATED $CO_2$ AS A CONVENIENT SOURCE OF CARBON DIOXIDE FOR ADULT MOSQUITO SAMPLING

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ABSTRACT. A new, convenient method was developed to supply $CO_2$ for mosquito sampling by using yeast, which converts sugar into $CO_2$ and ethyl alcohol. The system could, at average, generate 32.4 ml/min of $CO_2$ for at least 27 h. The total weight of the $CO_2$ generated was estimated to be 94 g. The efficacy of yeast-generated $CO_2$ as attractant for mosquitoes was significant, and the following 6 mosquito species were collected using yeast-generated $CO_2$ traps from July to September 2003 in a residential area of southern and northern Yokohama City, Japan: *Aedes albopictus* (Skuse), *Armigeres subalbatus* (Coquillett), *Culex halifaxii* Theobald, *Cx. pipiens pallens* Coquillett, *Ochlerotatus japonicus* (Theobald), and *Tripteroides bambusa* (Yamada). Besides mosquitoes, various other insects were collected in the trap. Species compositions of insects collected in yeast-generated $CO_2$ traps and dry-ice-baited traps were compared.

**KEY WORDS** $CO_2$, yeast, attractant, yeast-generated $CO_2$

## INTRODUCTION

Carbon dioxide is a mosquito attractant (Gillies 1980, Clements 1999) and has been used in various traps (Service 1993). In most of the previous studies, dry ice has been used as a source of $CO_2$. As an alternative $CO_2$ source, Hoy (1970) used $CO_2$ and CO fumes generated by an engine adapted to operate on liquid propane gas, and recently, some commercially available traps using that system have been developed (Burkett et al. 2001). However, $CO_2$ cylinders or generators are heavy and expensive, and thus have limitations, especially when trying to cover a wide area for mosquito surveillance. Dry ice is cheap and light, but in certain areas, like tropical countries, it is sometimes difficult to obtain.

We developed an alternative convenient method to supply $CO_2$ by using yeast, which converts sugar into $CO_2$ and ethyl alcohol. The idea of yeast-generated $CO_2$ as a source of carbon dioxide was first used in aquatic plant cultivation (Narten 1994). In aquatic plant cultivation, the length of the $CO_2$ supply period is most important, while for mosquito sampling, amount of $CO_2$ as well as the length of supply period are relevant. We conducted laboratory experiments to find a cheap and convenient method that would produce enough $CO_2$ for a long enough time to be used for mosquito collection. The efficacy of yeast-generated $CO_2$ as an attractant for mosquitoes was evaluated in field collections.

## MATERIALS AND METHODS

*Carbon dioxide production by yeast*: Figure 1 is a schematic picture of the yeast-generated $CO_2$ trap. Two plastic bottles (2-liter volume) were used to hold water solutions of sugar and yeast. The 2 bottles contained different concentrations of dry yeast and sugar: bottle A, 150 g of sugar + 12 g of dry yeast, and water added to total a volume of 1,500 ml; in bottle B, 100 g of sugar + 6 g of dry yeast, and water added to total a volume of 1,750 ml. Because bottle A contains a larger amount of dry yeast, the output rate of $CO_2$ is higher and the length of supply period is shorter than in bottle B. By using the 2-bottle system, we could achieve the high output rate of $CO_2$ as well as the long supply period. The bottles were connected to each other with polypropylene tubing and to a small (500-ml-volume) plastic bottle holding the overflowed water solution. Generated $CO_2$ was released from a 5-mm hole on the outer wall of the small bottle. For easy preparation of the water solution as well as cleaning, there were 3 joints in the connection tubing (Fig. 1). The small bottle was hung close to the opening of a suction trap, similar in design to the CDC-light trap (Service 1993). It was made of 14-cm-long acrylic tubing with an inside diameter of 8.5 cm attached to a 3.0-V motor driving a three-bladed plastic fan powered by four 1.5-V dry batteries.

*Measurement of yeast-generated $CO_2$*: The amount of $CO_2$ gas generated by the dry yeast was measured in the laboratory. The $CO_2$ gas released from the connection tube was accumulated into a bottle filled with water and the volume of $CO_2$ gas was measured every 3 h for 28.5 h. Because it took about 1–1.5 h to stabilize the output rate of $CO_2$ gas from the bottles, the measurement started 1.5 h after the initiation of the experiment. The experiment was replicated 5 times. Temperature condition during the experiment ranged between 25 and 27°C.

*Field evaluation of efficacy of yeast-generated $CO_2$ in mosquito collections*: Mosquito collections were conducted from July to September 2003 in residential areas of southern and northern Yokohama City, Japan. The efficacy of yeast-generated

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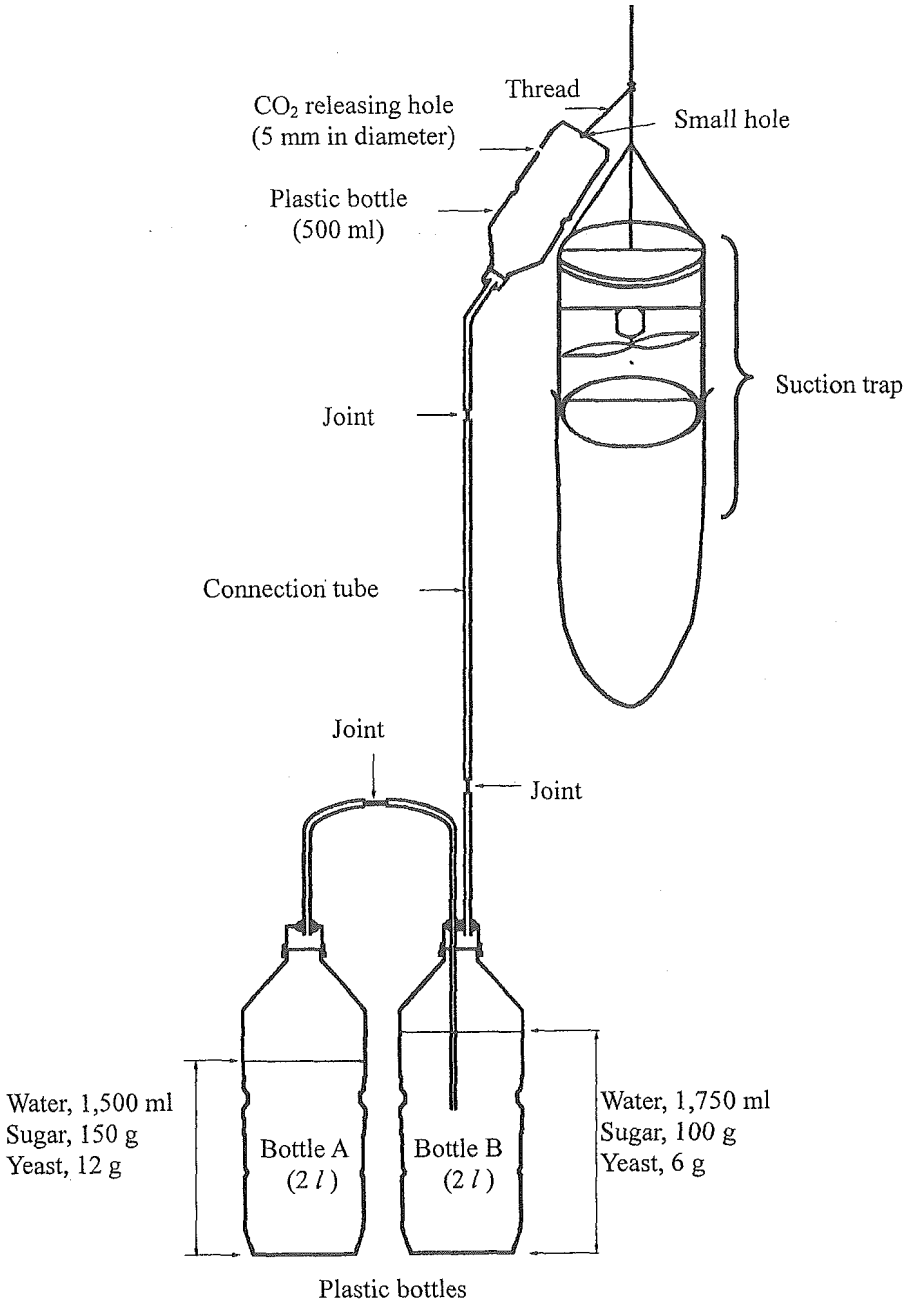


Fig. 1. Yeast-generated $CO_2$ trap. Carbon dioxide gas was generated inside plastic bottles A and B, with different concentrations of dry yeast and sugar, and released from the top of a suction trap through connection tubing and a small plastic bottle, which holds the overflowed water solution.

$CO_2$ was evaluated by 1) a comparison between suction-trap collections with and without yeast-generated $CO_2$ and 2) a comparison of trap collection between yeast-generated $CO_2$ trap and a dry-ice-baited (1 kg) trap. The first experiment was conducted 5 times in southern Yokohama City in August 2003. Two suction traps were operated for 24 h. The traps were placed 1.7 m apart, one of them enhanced with yeast-generated $CO_2$ and the other without $CO_2$. Mosquitoes collected were counted and species compared. The second experiment was conducted 4 times in northern Yokohama City, August–September 2003. The yeast-generated $CO_2$ trap and a dry-ice-baited trap were placed 4–5 m apart and operated for 24 h. The dry ice was wrapped with paper and placed in a styrofoam-box. A piece of dry ice always remained in the box after 24 h of collection. Collected insects were killed and counted, mosquitoes were identified to species, and other dipterans were identified to family.

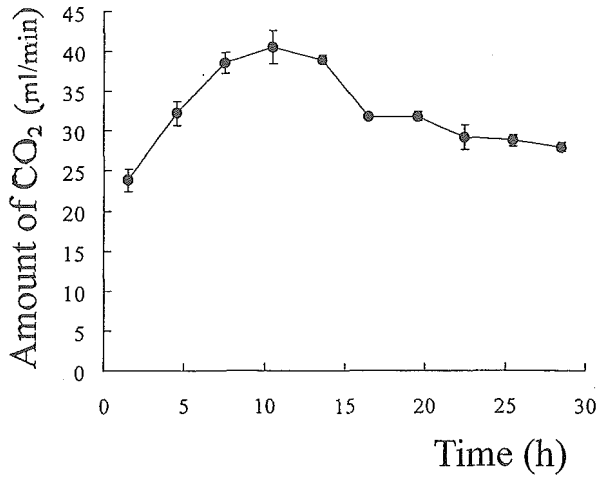


Fig. 2. Temporal changes in amount of $CO_2$ (mean ± standard deviation [SD]) generated by yeast under temperature conditions of 25–27°C.

## RESULTS AND DISCUSSION

The amount of $CO_2$ generated by the yeast increased during the first 10.5 h, reached a maximum output rate of 40.6 ± 2.1 ml/min, gradually decreasing (Fig. 2). At the end of the 28.5-h experiment, the output rate of $CO_2$ was 28.0 ± 0.6 ml/min. The mean output rate of $CO_2$ during the experiment (27 h) was 32.4 ml/min, nearly equal to the 30–40 ml/min $CO_2$ output rate of a chicken (Clements 1999). The total amount of $CO_2$ generated during the experimental period was about 52 liters. Assuming 1 atm and a mean temperature of 26°C during the experiment, the estimated weight of $CO_2$ generated was 94 g. In this study, tap water was used in the experiments. Preliminary observations showed that water collected from a pond (chemical oxygen demand = 4 ppm, pH = 7.3) and a river (COD = 2 ppm, pH = 6.6) could be used instead of tap water.

The collection was repeated 5 times and the number of mosquitoes collected in yeast-generated $CO_2$ traps was always larger (mean number = 18.0) than the number collected in traps without $CO_2$ (mean number = 1.0). Therefore, the efficacy of yeast-generated $CO_2$ as an attractant for mosquitoes was significant (sign test, $P = 0.031$).

The following 6 mosquito species were collected in yeast-generated $CO_2$ traps (Table 1): *Aedes albopictus, Armigeres subalbatus, Culex halifaxii, Cx. pipiens pallens, Ochlerotatus japonicus,* and *Tripteroides bambusa.* Both diurnal as well as nocturnal species were collected (Tanaka et al. 1979). The dominant species was *Cx. pipiens pallens* (253 ♀) followed by *Ae. albopictus* (56 ♀).

Besides these mosquitoes, various other insects were collected in the traps. The species composition of insects collected in yeast-generated $CO_2$ traps and dry-ice-baited traps is summarized in Table 2. The dominant mosquito species were the same in both yeast-generated $CO_2$ traps and dry-ice-baited traps: *Cx. pipiens pallens* and *Ae. albopictus.* The number of mosquitoes collected in yeast-generated $CO_2$ traps was smaller than in dry-ice-baited traps: 63 versus 103 *Cx. pipiens pallens* and 13 versus 24 of *Ae. albopictus* in yeast-generated $CO_2$ traps versus dry-ice-baited traps. Because the average output rate of $CO_2$ from 1 kg of dry ice was calculated as 387 ml/min, 12 times more than from yeast-generated $CO_2$, the difference in mosquito numbers may be largely ascribed to the difference in output rate between the yeast method and dry ice. Some differences in species composition were found, especially in the orders of Lepidoptera and Thysanoptera, between yeast-generated $CO_2$ traps and dry-ice-baited traps. Lorenzo et al. (1998) found that *Triatoma infestans* can be captured by yeast-baited traps. Because yeast converts sugar into $CO_2$ and ethyl alcohol, a certain amount of ethyl alcohol gas may also be released. Additional comparative experiments will be required to clarify the effects of the $CO_2$ and ethyl alcohol mixture on the species composition of the insects collected.

Table 1. List of mosquito species collected by a suction trap enhanced with yeast-generated $CO_2$ from 19 July to 19 August 2003, in southern Yokohama City, Japan.[1]

| Species | Female | Male |
|---|---|---|
| *Aedes albopictus* | 56 | 7 |
| *Armigeres subalbatus* | 4 | 0 |
| *Culex halifaxii* | 1 | |
| *Cx. pipiens pallens* | 253 | 6 |
| *Ochlerotatus japonicus* | 1 | 0 |
| *Tripteroides bambusa* | 11 | 1 |
| Total | 326 | 14 |

[1] Trap collection was conducted 12 times during the study period.

In this study, we used 2 plastic bottles (A and B) to hold the yeast and sugar solutions. For field surveys, it may be more convenient to use only 1 bottle. We conducted an additional experiment to compare the $CO_2$ output rate of the 2-bottle system with a 1-bottle system, in which the same amount of sugar (250 g) and dry yeast (18 g) as in the 2-bottle system was now kept in one 4-liter bottle. Three different amounts of water, 2.5 liter, 2.7 liter, and 2.9 liter, were used in the 1-bottle system and the mean $CO_2$ output rates during the first 7–24 h were calculated to be 33.6, 32.3, and 30.0 ml/min, respectively. There were no significant differences in $CO_2$ output rate between the 2-bottle system and the 1-bottle system (ANOVA, $F = 2.44$, $P = 0.26$). Therefore, the 1-bottle system can be expected to be as effective in attracting mosquitoes as the 2-bottle system at least for the first 24 h.

The amount of $CO_2$ generated by yeast depends on temperature, and thus, is affected greatly by seasonal changes, especially in temperate areas. To

Table 2. List of insects collected by suction trap enhanced with yeast-generated $CO_2$ or dry ice (1 kg) in northern Yokohama City, Japan, August–September 2003.[1]

| Order | Family | Species | Yeast | Dry ice |
|---|---|---|---|---|
| Diptera | Culicidae | *Ae. albopictus* | 13 | 24 |
| | | *Cx. pipiens pallens* | 63 | 103 |
| | | *Cx. bitaeniorhynchus* | 0 | 1 |
| | Cecidomyidae | | 23 | 12 |
| | Ceratopogonidae | | 9 | 4 |
| | Chironomidae | | 6 | 3 |
| | Chloropidae | | 1 | 0 |
| | Phoridae | | 0 | 1 |
| | Psychodidae | | 20 | 8 |
| | Sciaridae | | 6 | 4 |
| | Tipulidae | | 6 | 1 |
| Coleoptera | | | 1 | 2 |
| Hemiptera | | | 6 | 10 |
| Hymenoptera | | | 25 | 11 |
| Lepidoptera | | | 59 | 0 |
| Psocoptera | | | 4 | 3 |
| Thysanoptera | | | 0 | 36 |
| Total | | | 242 | 223 |

[1] The trap was operated for 24 h. Trap collection was made 4 times during the study period.

achieve a constant output rate of yeast-generated $CO_2$ gas throughout the year, a temperature-control system will be necessary in temperate areas. However, in tropical countries, temperature conditions are rather constant, so that our system will work well throughout the year.

Although the effect of $CO_2$ gas on the number of mosquitoes collected in suction traps is clear, it is usually difficult to obtain gas cylinders or dry ice in tropical areas, where mosquito-borne diseases are serious. The yeast-generated $CO_2$ trap developed in this study is convenient and cheap, and all the materials necessary are locally available. Our system might be valuable for *Ae. aegypti* surveillance in dengue-epidemic areas and malaria mosquito surveillance.

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Amino acid substitutions conferring insecticide insensitivity in *Ace*-paralogous acetylcholinesterase

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Since insecticide insensitivity of acetylcholinesterase (AChE) was found about 40 years ago as a cause of the resistance to organophosphates in the spider mite, more than 30 insect and acarus species have added to the instance. Based on the 3-dimentional analysis of *Torpedo* AChE structure and sequencing of *Drosophila* AChE gene (*Ace*), amino acid substitutions conferring the insensitivity has been found in *Drosophila melanogaster*. However, no amino acid substitution responsible to the AChE insensitivity had been found in insects and acari except Brachicera flies until the second type of AChE paralogous to *Ace* was discovered in *Schizaphis graminus* and *Anopheles gambiae*. Sequencing of *Ace* -paralogous AChE cDNAs has followed in insect species of various orders. Now, various amino acid substitutions are found and corresponded to different biochemical properties of insensitive AChEs in relation to the function of substituted amino acids in the 3-dimentional structure. Existence of two AChE genes raises questions about differentiation of the two genes, site of gene expression, and function of each enzyme.

Key word: acetylcholinesterase, *Ace*-paralogous, insecticide resistance, insensitivity, amino acid substitution

## 1. Introduction

The altered acetylcholinesterase (AChE) is an important resistant mechanism as well as other insecticide-target molecules, GABA receptor [1] and sodium ion channel [2,3]. In the target insensitivity, it appeared that the substitution of amino acid residues occurs at the conserved positions of the structural protein in a wide range of species from different insect orders. The position of the substitution is located in the proposed important site of sodium ion channel [2,3]. The insensitivity of AChE accompanies also amino acid substitutions and has been reviewed timely [4,5,6,7,8]. In this review, the recent progress on molecular mechanism of insensitivity to organophosphates and carbamates is described especially concerning an insect AChE family that is paralogous to *Drosophila* AChE (*Ace*).

The insect AChE gene was first sequenced in *Drosophila melanogaster* [9] and several amino acids substitutions conferring insecticide insensitivity was also first elucidated in this species [10] followed by Brachycera fly species, *Musca domestica* [11,12,13], *Lucilia cuprina* [14], and *Bactrocera oleae* [15]. However, there found no amino acid substitution in the *Ace*-orthologues of other insects whose AChE insensitivity had been well characterized [4], because genome data of *D. melanogaster*, a model of insects, showed

that only one AChE gene exists in its genome [16]. Several years had passed without progress, when studies on the mechanism of AChE insensitivity were restarted by the discovery of the second AChE genes paralogous to *Ace* in insects [17,18].

Names of insect AChE are now confused since the second AChE gene was discovered. After the discovery of the new AChE gene, two types of AChEs have been named freely by the workers. In order to avoid confusion, we use AO-AChE and AP-AChE for *Drosophila Ace*-orthologous and -paralogous gene family members, respectively in the text.

**Structure of AChE**

Acetylcholinesterase (AChE, EC 3.1.1.7) terminates synaptic transmission at cholinergic synapses by hydrolyzing excess acetylcholine (ACh) released from the presynaptic membrane. The inhibition of AChE by organophosphate and carbamate insecticides accumulates ACh in the synaptic gap and causes a desensitization of the ACh receptor, leading to a blockage of the signal transmission. Insect AChE is a homodimeric globular protein of about 150 kDa linked to membranes by its C-terminal end with a glycosyl-phospatidil-inositol anchor. Dimeric subunits are linked covalently by a disulfide bond. The protein is expressed as a precursor that is subsequently glycosylated, processed at its C-terminal for the removal of hydrophobic peptide extension by glycolipid anchor replacement.

Crystallographic analysis of dimeric AChE of *Torpedo californica* presented its three-dimentional structure [19]. The schematic drawing of AChE active site with ACh is shown in Fig. 1. The active site lies near the bottom of a deep and narrow gorge that reaches halfway into the protein. The gorge is lined with 14 highly conserved aromatic residues that have the role in facilitating diffusion of the substrate to the active site. There is a catalytic triad, Glu327, His440, and Ser200, with appropriate hydrogen bonding distances and alignment in the active site. The triad involves a dicarboxylic amino acid with drawing a proton from a serine through the imidazole of His. Oxyanion hole next to the triads is composed of Ala201, Gly118 and Gly119 that stabilize the carbonyl oxygen of ACh through hydrogen bonding. A clear delineation of the acyl pocket is composed of the side chains of Trp233, Phe288, and Phe290 pointing inward toward the binding site. These residues would be expected to constrain the dimensions of the acyl pocket in AChE. The choline moiety appears to be stabilized by Trp84 situated at choline binding site in AChE whose orbitals lie close to the trimethyl ammonium surface, as defined by its van der Waal's radii. The X-ray analysis of *Drosophila Ace* [20] showed that the three-dimensional structure is similar to that of *T. calfornica* in its overall fold, charge distribution, and deep active site gorge. The active site gorge of Ace having 9 aromatic amino acids in its surface is narrower than that of *Torpedo*, and subsequently the volume of the lower part of the gorge is less than 50% of *Torpedo*. In *Drosophila* Ace, the acyl pocket is composed of Trp309 (equivalent to 233 amino acid in *Torpedo* AChE), Phe368 (290), and Phe478 (400) which is used as a component instead of Phe288 of *Torpedo* AChE (Fig. 2) [20].

**AChEs in resistant insects and their biochemical properties**

Organophosphates and carbamates have analogous structure to the substrate of ACh and inhibit competitively AChE at the active site. Hydrolysis of these inhibitors leads to an enzyme with phosphorylated or carbamylated active serine, and then retards excessively the reactivation of the enzyme.

The insensitivity of AChE to organophosphates was first reported in the two-spotted spider mite, *Tetranychus urticae*, associated with organophosphate resistance [21]. Biochemical properties of the insensitive AChE suggested that some modification occurred at the active site of enzyme [22]. Similar biochemical investigations also showed that more than 30 insect and acarus species made their AChEs insensitive to the organophosphates and carbamates by the modification of substrate binding site [9]. However, appearances of insensitivity to inhibitor and catalytic activity of substrates are specific to insect species.

In the resistant Toyama strain of *Cx. Tritaeniorhynchus*, insensitivity of AChE is more than 1000 times to most of organophosphates and 100 times to carbamates compared with the susceptible Taiwan strain (Fig. 3). The AChE in the resistant strain shows no optimum pH and no optimum substrate concentration, while higher substrate specificity to ACh. When the acetylthiocholine (ATCh) was used as a substrate, the hydrolytic activity of the Toyama strain declines to one third that of the susceptible strain (Table 1). Since the structural difference between ACh and ATCh lies in the length of the molecule binding to the active site, it is suggested that a substantial structural modification has occurred at the site [23]. Quantitative structure activity relationship analysis of AChE in the two strains using 3-alkylsulfonylphenyl methanesulfonates confirmed that the length of 3-alkyl moieties which interact with choline binding site does not affect the insensitive ratio. A parabolic relationship of the activity was detected with the distance between the S atom and the distal C atom of the alkyl moiety. The shape of the parabolic curve and the optimum distance (4.55 A) were the same in susceptible and resistant strains, but the inhibitory activity was about 100 times higher for AChE of the susceptible strain than for that of the resistant strain (Fig. 4) [24].

AChE of resistant *Tetranychus kanzawai* showed similar property with *Cx. tritaeniorhynchus*, higher insensitivity to organophosphates than to carbamates, and reduction of hydrolyzing activity to artificial substrates of longer chain [25]. In *Cx. pipiens*, AChE of resistant strain showed very high insensitivity to a carbamate, propoxur, and significant decrease of hydrolytic activity to ATCh [26,27]. AChE of the resistant strain (Nakagawara) of the green rice leafhopper, *Nephotettix cincticeps*, showed different inhibition appearances from those mentioned above [28,29]. It is insensitive to carbamates, propoxur by 115 times and to other monomethyl carbamate by 50 times or less compared with normal AChE. To the contrary, it is 3-10 times more sensitive to *n*-propyl carbamates and some organophophates such as propaphos-sulfoxide, diazoxon, and pyridafenoxon than normal one. As for substrate specificity of the insensitive AChE in *N. cincticeps*, hydrolytic

activity for ATCh was not changed but no optimum substrate concentration was found [29]. AChE of resistant strains of aphids, *Myzus persicae* [30,31] and *Aphis gossypii* [32,33] showed unique appeareances of insensitivity. It is insensitive to dimethyl carbamates such as pirimicarb and rather sensitive to some monomethyl carbamates and organophosphates. Another type of AChE insensitivity found in *A. gossypii* [34] shows higher insensitivity to organophosphates.

Quantitative activity structure relationship in *N. cincticeps* using 6-alkylthio-2-pyridyl methanesulfonates indicated different appearances of AChE from *Cx. tritaeniorhynchus* [35]. A statistically significant parabolic relationship was obtained between the steric constant of the alkyl moiety and the inhibitory activity, but not for other structural parameters. Inhibitory activity was maximized when the steric constant was -1.26 for the susceptible strain, and -1.66 for the resistant strain. These results suggest that the inhibitory activity apparently changes according to the bulkiness of the alkyl moiety and the AChE of resistant strain is inhibited by methansulfonates with more bulky moieties compared to susceptible one.

**Discovery of Ace paralogous AChE (AP-AChE)**

Molecular studies revealed that the insensitivity of AChEs was accompanied by some amino acid replacements in Brachycera fly species. However, no insensitivity-specific mutation was successfully identified in the AO-AChE transcripts from resistant strains of *Cx. pipiens* [36], *Cx. Tritaeniorhynchus* [37], *M. persicae* [38], *A. gossypii* [39,40], *N. cincticeps* [41], *Plutella xylostella* (Terada unpublished) and *Oulema oryzae* (Tomita, unpublished). In *Cx. tritaeniorhynchus*, both the structural gene locus for AO-AChE and OP insensitive trait locus of AChE ($AChE^R$) were mapped with RFLP markers by a back cross QTL (quantitative trait loci) analysis. A single major locus for $AChE^R$ was identified on chromosome 2, while AO-AChE (AChE1) locus was directly mapped to chromosome 1 by using AChE cDNA probe (Fig. 5) [42]. These results are completely consistent with previous findings [43] that the $AChE^R$ locus maps to chromosome 2. The $AChE^R$ locus in *Cx. tritaeniorhynchus* seemed to encode either another AChE isoform or an undefined biomolecule that interacts directly with AChE to promote conformational changes and results in insensitivity to inhibitors. The literature reflects conflicting support for both scenarios. Analogous result was obtained in *Cx. pipiens*, in which a single AChE gene has been identified that is located on chromosome 1, while the $AChE^R$ locus maps to chromosome 2 [36]. The primary support for the putative existence of two unlinked AChE genes is provided by *Cx. pipiens* where two electrophoretically distinct isoforms of AChE have been identified, only one of which seems to be involved in AChE-mediated insecticide resistance [44]. However, only a single AChE enzyme is evident in all other mosquitoes examined to date, including *Ae. aegypti*, *Anopheles gambiae*, *An. stephensi*, *Culiseta longeareolata* and *Cx. hortensis* [45]. Support for the existence of a genetic post-translational modification that influences AChE activity through conformational

changes comes largely from the inability to identify a second AChE gene in any mosquito species.

A breakthrough in this toxicological riddle was achieved in 2002. An AP-AChE cDNA was cloned from the greenbug *Schizaphis graminum* [17] and its putative homolog was identified following genome sequence determination in the mosquito *An. gambiae* [18,46]. Since these determinations, AP-AChE cDNA sequences have been reported for insects including the mosquitoes *Cx. pipiens* [47], *Ae. aegypti* (GB: Accession No.AJ428049), *Ae. albopictus* [Mizuno, unpublished] and *Cx tritaeniorhynchus* [37], aphids *A. gossipii* [48] and *M. persicae* [38], the leaf hopper *N. cincticeps* [Terada, unpublished], moths *Plutella xylostella* [51] and *Bombyx mori* [Kazuma, unpublished], the honey bee *Apis merifera* (XP-393751)))))), and the lice *Pediculus humanus* [Tomita, unpublished]. In *Cx. tritaeniorhynchus*, the AP-AChE cDNA sequence encoding complete coding sequence of enzyme precursor was determined by primer walking (Fig.2), initially based on conserved peptide sequences of AP-AChEs from *S. graminum* [17] and *An. gambiae* [18]. The AP-AChE precursor includes a putative 42 amino acid signal peptide and a vertebrate H-peptide like segment. All of the common features of AChE are conserved, the catalytic triad (Ser325, Glu451, and His565), the 6 Cys residues for forming 3 intra-subunit disulfide bonds, a Cys for inter-subunit disulfide bond, the oxianion hole, and 12 out of the 14 aromatic residues lining the active site gorge of *T. californica* (Fig. 2). As for the acyl pocket, Phe455 (331) was added to component amino acids, along with Trp358 (233) and Phe424 (290) by the three dimensional modeling of AChE structure [50,37].

A molecular phylogenetic tree involving currently available insect AO- and AP-AChEs whose registered sequences nearly cover the expected mature protein sequences was constructed and is shown in Fig. 5. In this tree, two insect AChE subfamilies consisting of AO- and AP-AChEs, are clearly separated. Homology of protein sequence between AO-AChE and AP-AChE of *Cx. tritaeniorhynchus* is 40% (identity of amino acids), and AP-AChEs of *Cx. tritaeniorhynchus* shows 93% and 64% to AP-AChEs of *An. gambiae* and *M. persicae*, respectively. AChEs found in *T. urticae* [51] and *T. kanzawai* [52] are rather closely related to AP-AChE subfamily.

According to *Eco*RI RFLP analysis using the endogenous cDNA probes for AP-AChE genes, the AP-AChE locus of *Cx. tritaeniorhynchus* was mapped to a region within 0.2 cM of the $AChE^R$ phenotype on chromosome 2 (Fig.6) [37]. The result indicates that the $AChE^R$ is identical with AP-AChE gene, and that only mutation(s) in the gene are associated with the insensitive AChE phenotype.

**AA substitution in AP-AChE conferring insensitivity**

Several different positions have been pointed out for the amino acid substitution conferring insecticide insensitivity of AP-AChE. One is Gly to Ser at the oxyanion hole in *Cx. pipiens* [18], *An. gambiae* [47] and *An. albimanus* [53], and the second is Phe to Trp in *Cx. tritaeniorhynchus* [37] or Ser to Phe in *M. persicae* [38] and *A. gossypii* [39,40] at the

acyl pocket as shown in Table 2. The amino acid substitution Gly280(119)Ser (Gly 280 is replaced by Ser. The number of the equivalent amino acids in *T. californica* AChE is shown in parenthesis.) in *Cx. pipiens* is responsible for an extraordinary level of propoxur insensitivity [18]. In *Cx. tritaeniorhynchus*, the substitution Phe455(331)Trp was found to be accompanied by high insensitivity of AChE to most organophosphates and carbamates [37]. Ser431(331)Phe substitution in *A. gossypii* [39,40] and *M. persicae* [38] correlates pirimicarb insensitivity. This substitution for Phe gave, to the contrary, no organophosphate insensitivity or rather higher sensitivity to organophosphates such as dichlorvos to the aphid AP-AChE [36, Nabeshima unpublished data]. In *A. gossypii*, Ala300(201), another amino acid in oxyanion hole, is substituted to Ser together with Ser431(331)Phe in the AP-AChE being insensitive to organophosphate [39,40,54]. In *Tetranychus* mites, the AChE of susceptible strain has Phe439(331) which is substituted with Trp and with Cys in the resistant strain of *T. kanzawai* [52] and *T. urticae*, respectively [51]. Recently, another amino acid substitution in the acyl pocket was found in *N. cincticeps*. The substitution Phe349(290)Val is considered to cause carbamate insensitivity in AP-AChE of this species (Terada, unpublished). In *P. xylostella* AP-AChE, Gly(227)Ala substitution which is equivalent to one of substitutions conferring organophosphate insensitivity of AO-AChE in *D. melanogaster* and *M. domestica* was pointed out for its prothiophos insensitivity [49].

To evaluate the effect of the mutation on biochemical characteristics of AP-AChE, the catalytic properties and insecticide sensitivity were compared between wild-type and mutant recombinant AP-AChE that was expressed *in vitro*. The mutant AP-AChE of *Cx. pipiens* with Gly280(119)Ser expressed in S2 *Drosophila* cells showed the same level of insensitivity to propoxur as AChE of resistant strain. As for AChE of *Cx. tritaeniorynchus*, AP-AChE cDNAs with Phe455(331) (wild type, AP-CxTS) and with Trp455(331) (Mutant, AP-CxTI), and AO-AChE (AO-CxT) were expressed in a baculovirus-insect cultured cell system, and their biochemical properties were determined to evaluate the effect of the substitution on insensitivity [55]. *Km* values of AO-CxT, AP-CxTS and AP-CxTI are presented in Table 3. Comparing the hydrolyzing activity for the natural substrate, ACh, between AO-CxT and AP-CxTS, AP-CxTS showed three times higher affinity to the substrate than AO-CxT, but similar affinity to other artificial substrates, ATCh, PTCh and BTCh. Compared to AP-CxTS, AP-CxTI which has Phe455Trp substitution, showed greater Km values for ATCh, PTCh and BTCh (7.04 times, 10.50 times and 20.75 times, respectively) showing very low affinity to these substrates, while the affinity to ACh was maintained to be high (0.83 times to AP-CxTS ). When the sensitivity of three AChEs were compared for five inhibitors (Table 4), AO-CxT was rather sensitive to these inhibitors than AP-CxTS, especially DDVP for which sensitivity of AO-CxT was about 30 times higher. $I_{50}$ values of AP-CxTI for fenitroxon and DDVP indicate 12,400 and 3,300 times higher insensitivity than that of AP-CxTS, respectively. For monomethyl carbamates, carbaryl and eserine, the $I_{50}$ ratio of AP-CxTI to AP-CxTS were 180 times and 750 times,

respectively. These results indicate that the reduction of sensitivity by the mutation is greater for organophosphates than for monomethyl carbamates. The substitution Phe455(331)Trp suggests the acyl pocket dimension to be smaller and explains well the biochemical data though the Phe455(331)Trp replacement changed also its electrostatic field. By the aspect of the dimension of acyl pocket, Ser431Phe substitution at the equivalent position in aphid species, *Myzus persicae* [38] and *Aphis gossypii*, [39,40] seems to make the acyl pocket smaller than that with Ser, and to explain the reduction of sensitivity to pirimicarb and increased the sensitivity to certain carbamates and organophosphates.

Recombinant AP-AChE of *Cx. tritaeniorhynchus* with amino acid substitution, Gly245(119)Ser as in *Cx. pipiens*, Ala326(201)Ser as in *A. gossypii* and *M. persicae*, and Phe414(290)Val as in *N. cincticeps* were also expressed in baculovirus-insect cell system. These substitutions reproduced the specific inhibition properties of each substitution even in the heterogeneous background. Recombinant AP-AChE with Gly245Ser was greatly insensitive to carbaryl, a monomethyl carbamate, and moderately insensitive to fenitroxon and DDVP, that with Ala326(201)Ser showed insensitivity only to pirimicarb, a dimethyl carbamate, and that with Phe414(290)Val was insensitive to carbaryl, slightly insensitive to fenitroxon and DDVP, and sensitive to propaphos sulfon, a di n-propyl phosphate (O, unpublished).

**Expression of two AChE genes**

There have been reported several studies on the expression of AP-AChE. Nothern blot analysis indicated that AP-AChE expression was 1.5 fold higher in the resistant strain of *S. graminum* than in the susceptible strain [56]. In *Boophilus microplus*, RT-PCR analysis showed that AChE1 is expressed in salivary glands and ovaries as well as synganglia [57]. Since the most insects except Brachycera flies appeared to have two types of AChEs, it is necessary to investigate precisely the expression of the two AChE isoforms in order to elucidate their distribution and function. Expression of the two AChEs was measured by quantitative real-time RT-PCR in *P. xylostella*, and showed that the transcription level of AP-AChE was 100-220 and 100-250 fold higher than that of AO-AChE in the adult and larval head, respectively [49]. In *Pediculus humanus* whose AP-AChE and AO-AChE cDNAs were sequenced recently, two AChEs showed a rather comparable expression level in the adult, ratios of AP-AChE to AO-AChE transcripts in head and body without head, 3.1 and 9.3, respectively [Tomita, et al. unpublished]. Expression level of the two AChE genes was also compared through the developmental stages, embryo to adult, in *Ae. albopictus* (Mizuno, unpublished). AO-AChE gene expression level greatly fluctuated at lower level from embryo to adult compared to that of AP-AChE gene. The ratios of AP-AChE to AO-AChE transcripts were 3.5 and 9.5 in adult head and whole adult, respectively. The expression level of AO-AChE was very low at second and third larval instar, and the ratio of AP-AChE to AO-AChE was 300.