REVIEW ARTICLE

Biocontrol efficacy of selected mosquitocidal bacteria

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Manuscript details:

Received: 25.10.2017
Accepted: 13.11.2017
Published : 05.12.2017

Editor:
Dr. Arvind Chavhan

Cite this article as:
Sivakami V, Kannan S, Rajendran J and Selvaraj Pandian (2017) Biocontrol efficacy of selected mosquitocidal bacteria.; *International J. of Life Sciences*, 5 (4): 577-786.

ABSTRACT

Insect species represent the largest percentage of the world's known species. They are undoubtedly the most adaptable life forms existing on the Earth. Less than 0.5 percentage of the total number of the known insect species are considered as pests. Among these, mosquitoes pose a major threat to public health by transmitting diseases like malaria, dengue, chikungunya etc. Only four classes of chemical insecticides have been approved by WHO with less target sites. Indiscriminate use of these synthetic insecticides and resultant selection pressure on insect populations has caused many mosquito species remain resistant to widely used insecticides. Hence, alternative approach has been initiated to use biological agents. This biocontrol strategy is important in order to counter the evolution of resistance in target populations and possible effects on non target organisms. Unlike chemical insecticides, biocontrol agents are host specific, safer to the environment, find easy application in the field, long-lasting effect with single application and cost effective production. In this context, this paper reviews about the important mosquitocidal bacteria and their efficacy against different mosquito species.

Keywords: Mosquitocidal, Insecticides, resistance, biocontrol agents

INTRODUCTION

Mosquitoes are considered as large group of insects present throughout the temperate and tropical regions and even beyond the Arctic Circle of the world (Harbach, 2007). India is ranked fifth in terms of mosquito biodiversity after Brazil, Indonesia, Malaysia and Thailand (Foley *et al.,* 2007). They belong to family Culicidae, order Diptera and are divided into two subfamilies and 112 genera. At present, a total of 3,540 recognized mosquito species are recorded in the world. Among this, the Indian mosquito fauna includes 393 species which is divided among 49 genera and 41 subgenera. Most of the important disease vectors are the members of Anopheliae and Culicidae. In India, 31 species are currently recognized for

transmitting various mosquito-borne pathogens (Bhattacharya *et al.,* 2014). They act as vectors to transmit most of emerging diseases such as malaria, yellow fever, dengue, chikungunya, filariasis, encephalitis, West Nile fever etc.

Vector borne diseases-Global burden

Vector-borne diseases are responsible for 17% of the global burden of parasitic and infectious diseases (WHO, 2008). Within the past two decades, many important vector-borne diseases have re-emerged or spread to new parts of the world. Traditionally, it is regarded as a problem of tropical countries; now pose an increasingly wider threat to global public health, both in terms of the number of people affected and their geographical spread (WHO, 2014). For example, some of the vector borne diseases such as dengue, chikunguniya and West Nile virus are emerging in the countries where they are previously unknown. This is mainly due to seasonal weather variation, socio-economic status, vector control programmes, environmental changes and drug resistance which are highly like to influence current vector-borne disease epidemiology. These effects are likely to express in many ways from short term epidemics to long-term gradual changes in disease trends (Githeko *et al.,* 2000).

Vector control strategies:

The control of mosquito borne diseases remain a major problem due to the absence of effective vaccines or specific anti-viral drugs. Vector control is a powerful preventive tool that is not used to its full potential. It is defined as measures of any kind, directed against vectors of diseases and intended to limit their ability to transmit diseases (Karunamoorti, 2013). Mosquito control is carried out mainly with chemical insecticides such as organophosphates, carbamates and pyrethroids. Synthetic chemical insecticides such as DDT, delmethrin, malathion, chloropyriphos, etc are popularly used as first line of defense against pest populations particularly mosquito vectors. In 1955, WHO proposed the eradication of most of the prevalent vector-borne disease Malaria with the use of residual house-spraying of DDT (Hemingway and Ranson, 2000). The utilization of these products has been limited because they are non-specific, pollute the environment and their target insects have high rates of resistance.

Synthetic insecticides:

World Health Organization has approved a list of synthetic insecticides which are used commercially to treat adult mosquitoes to date. They have been categorized under four different classes which include organochlorines (now banned in most countries), organophosphates, carbamates and pyrethroids (Zaim and Guillet, 2002). Only a limited number of insecticide classes are available for adult mosquito control. No new malaria mosquito adulticide has been approved by the WHO in the last 15 years (Nauen, 2007). It is important to note that these four chemical classes of insecticides possess only two different modes of action indicating less target diversity when compared to agricultural pesticides (Nauen and Bretchneider, 2002). Moreover, the use of chemical insecticides results in undesirable effects such as increased physiological resistance in vectors, environmental pollution lead to bio-amplification in food chain and killing of non-target populations such as earthworms, birds etc.

Insecticide resistance:

Resistance is defined as the developed ability in a strain of insects to tolerate doses of toxicant which would prove lethal to majority of individuals in a normal population of the same species (WHO,1957). The resistance to insecticides is considered to be a recent evolutionary adaptation in insects which occurs in less than one century with response to sequential application of insecticides. The possible mechanisms to develop resistance is to enhance the ability of insects to detoxify the insecticide molecules and to alter the target sites so that insecticide molecules no longer bind with the action sites (Brattsten, 1986).

The emergence of resistance act as major hurdle in the line of current vector control programmes. More than 40 years of intensive synthetic insecticides use to control arthropod pests and disease vectors have resulted in pesticide resistance among over 450 species (Georghiou, 1986). Resistance is commonly monitored by bioassay either by determining LC50 value or by using uniform diagnostic doses (Feng Cui *et al.,* 2006). Susceptibility studies of malaria vectors *A.stephensi Liston* and *A.subpictus Grassi* , collected from different locations in arid and semi-arid regions of India are conducted by adulticide bioassay of DDT, malathion, deltamethrin and larvicide bioassay of fenthion, temephos, chloropyriphos using diagnostic doses. Both the *Anopheline sp*. showed variable

resistance to DDT and malathion, larvae of *Anopheline sp. s*howed resistance to chloropyrophos followed by fenthion (Tikar *et al.,* 2011). In recent years, the knowledge of resistance status is essential to select a particular insecticide against target species in vector control programs. Hence, Worldwide Insecticide resistance Network (WIN, http://win-network.ird.fr) collaborates with internationally recognized institutions in vector research to track insecticide resistance at a global scale. The objective of WIN is to provide WHO and member states to evidence and expertise resistance management and deployment of alternative arbovirus vector control measures (Corbel *et al.,* 2016). In order to control human disease vectors, there is a need for alternate, more effective and environment-friendly control agents which enable long term sustainable results.

Biological agents:

The balance of nature depends to a large extent on the regulation of population densities by parasitoids, predators, competitors, parasites and pathogens. These natural enemies play an important role in checking the proliferation of vectors in nature. In this aspect various biological control agents have been thoroughly investigated with the support of World Health Organization Special Programme for Research and Training in Tropical Diseases(WHO/TDR) (Mulla, 1990). A large number of mosquito pathogens and parasites have been isolated and studied for the bio control of mosquitoes.The term biological control is defined as the control of pests, including the vectors of human disease by the direct or indirect use of natural enemies with or without their metabolites(WHO, 1982).The first biocontrol was *Bacillus popilliae*, entomopathogenic bacteria which was used against larvae of Japanese beetle. It was the first bacterium registered as insecticide in United States (Zhang *et al.,* 1997). Processed formulations were applied into soil and the pest population remains suppressed for more than 10 years after one application.

Entomopathogenic Bacteria:

Insect pathogenic bacteria are present in the families Pseudomonadaceae, Enterobacteriaceae, Lactobacillaceae, Micrococcaceae and Bacillaecae. In the past few decades, several bacterial isolates and strains of spore forming bacteria have been isolated that produce parasporal proteins which show high toxicity against insects (Katara *et al.,* 2012). Based on the safety to non-target organisms, only members of Bacillaceae (Order: Eubacteriales) were the most studied, commercialized, and successfully used in microbial control of lepidopteron, dipteran and coleopteran insect pests (Lacey *et al*.,1986). Among these, two bacteria such as *Bacillus thuringeinsis serovar israelensis (Bti)* and *Bacillus sphaericus (Bs)* have been successfully tested against mosquito larvae. There are certain guidelines for laboratory and field testing of mosquito larvae and it remain as universal method to test any biocontrol agent (WHO, 2005).

Bacillus thuringiensis israelensis(Bti):

Bacillus thuringiensis is a gram positive, rod shaped, spore forming bacterium characterized by its ability to exhibit insecticidal properties. This bacillus crystalline inclusions dissolve in the larval midgut, releasing one or more insecticidal crystal proteins (also called delta endotoxins) of 27 to 140 kilodaltons(kDa) (Hofte and Whitely, 1989). This appears to be a synergistic interaction between four proteins resulting in a highly complex mode of action which lead to the toxicity of mosquito larvae and with no resistance development. The *Bti* spores and parasporal crystals must be ingested by the larval (feeding) stage of target organism to cause mortality. The toxin binds to a receptor on the midgut cell wall resulting in pore formation in the cell and leading to death of the larva. *B. thuringiensis* was found to induce cellular and oxidative stress prior to mosquito death (Ahmed, 2013).

Bti is highly pathogenic against mosquitoes (*Culicidae*) and black flies (*Simuliidae*) and has some virulence against certain other *Diptera* especially *Chironomidae (*midges). Previouly, formulations of *Bacillus thuringiensis* have been used successfully as biocontrol agent to control agricultural pests, but their role in control of dipteran species was recognized only after the discovery of *B.thuringiensis* serovar *israelensis (Bti).* In 1975-76 under a World Health Organization sponsored project, a new *Bt* strain was discovered in Israel by Goldberg and Margalit (1977). This strain was isolated from *Culex* sp. dead larvae mosquito. Later, it was identified as *Bt israelensis*, serotype H14 according to its flagellar antigenicity. As a result of extensive research on the efficacy and evaluation of agents, *B.thuringiensis* (H-14) was effective in the field and registered for mosquito control in 1980 (Mulla *et al.,* 1984).

Table 1: List of *Bacillus thurigiensis israelensis* strains reported as mosquitocidal bacterial strains

Bacillus thuringeinsis israelensis	**Selected references**	**Source of isolation**
B.t jegathesan	Seleena and Lee,1990; Seleena *et al.,*1995	Malaysia(soil)
B.t medellin	Orduz *et al.,*1992; 1996; Thiery *et al.,*1996,	Colombia(soil)
B.t jegathesan	Seleenaand Lee,1990, Seleena *et al.,*1995	Malaysia(soil)
B.t sotto	Ohba *et al.,* 2000; Ohugushi *et al.,*2003	Okinawa, Japan(soil sample)
B..t. fukuokaensis	Ohba and Aizawa,1990; Lee and Gill, 1997; Guerchicoff *et al.,*1997	Japan
B.t.kyushaensis	Ohba and Aizawa, 1979; Held *et al.,*1990	Japan (*B.morri* breeding site)
B.t.israelensis	Goldberg and Margalit,1977	Israel(sewage pond)
B.t. morrisoni	Padua *et al.,* 1984	Phillippines(soil sample)
B.t. darmastadensis	Padua *et al.,*1980,	Japan
B.t. canadensis	Ishi and Ohba,1993	Iraq(soil)
B.t. thompsoni	Manonmani and Hoti,2001	India(soil)

More than 40,000 species of *Bacillus thuringiensis* have been isolated and identified which belongs to 39 serotypes. These include strains with various serotypes such as *Bt.canadensis*, *Bt.thompsoni*, *Bt.malaysiensis* and *Bt.jegathesan*. Among these, *Bt.medellin* and *Bt.jegathesan* appear as good candidates for further characterization and investigation (Ragni *et al.,* 1996).

These organisms are active against either *Lepidoptera*, or *Diptera* or *Coleoptera. Bti* was found to be specific toxic to larvae of 109 mosquito species *Bti* has an LC50 in the range of 10–13 ng/ml against the fourth instar of many mosquito species (Federici *et al.* 2003). Generally, *Culex* and *Aedes* are highly susceptible compared to *Anopheles* which are less susceptible (Balaraman *et al.,* 1983). Much higher concentrations of *Bti* are required to induce mortality in anopheline larvae than in *Aedes* species.

Limitations:

B.t.i formulations produced commercially are not active against adult flies, though the proteins in the parasporal body can be able to destroy the midgut epithelium of adults. This is mainly due to inability of proteins to penetrate the cuticle. There are no available methods to induce adult flies to ingest formulations under field conditions. Therefore, *Bti.* formulation commercially available at present are used as larvicides not as adulticides. It is well known that the toxicity of *Bti* lasts only a few days at most and efficacy can be reduced within 24 hours (Becker *et al.,* 1993). In addition, *Bti* does not survive long in highly polluted water and is particularly prone to UV light inactivation in strong sunlight (Mulla, 1990).

Most of these formulations offer high levels of initial control, but with very little residual activity. This has necessitated weekly application of these formulations to keep the larval population under constant check, which would increase logistics and cost (King *et al.,*1997). Therefore, a new formulation with long residual life and new mode of action is necessary. Due to high cost of production compared to chemical pesticides, operational success of *Bti* against the three major vectors is only limited to temperate regions (Europian countries) of the world, where these vectors are considered as nuisance pests (Porter *et al.,* 1993).

Bacillus sphaericus(Bs):

Bacillus sphaericus is another most extensively studied spore forming bacterium for its mosquitocidal properties. During sporulation, the active strains produce crystal toxin which is a binary toxin. The 51 and 42 kDa mosquitocidal crystal proteins of *B.sphaericus* are unique among bacterial insect toxins which have a low sequence similarity and are distinct from all of the cloned and sequenced insect toxins of *Bacillus thuringiensis* (Baumann *et al.,* 1991). Upon ingestion of this toxin by mosquitoes, they bind to specific receptors present in the midgut brush-border membrane and cause damage to midgut cells and lead to death. The first reported *Bacillus sphaericus* was not effective strain but after the isolation of *B.s* from

Indonesia (strain 1593) which is highly mosquitocidal (Charles *et al.,* 1996).Currently nine serotypes are known to contain active strains of *Bacillus sphaericus*. *Bacillus subsp isrealensis* and *B.sphaericus* differ in the nature of toxin and their host range.

In general, *B.sphaericus* is more effective against *Culex* spp and *Anopheles* but less effective to *Aedes* spp. *B.isrealensis subsp.* remain effective against *Aedes and Culex* spp but not to *Anopheles* spp *(*Lacey and Undeen,1986, Mulla, 1990*)*. In addition, *B. sphearicus* has its ability to survive in polluted aquatic environments but *B.ti* used to lose its ability in that environment (Mulla *et al.,* 1984, Davidson *et al.,1984,).* Most of mosquitocidal *B.s* strains were isolated successfully for the past 30 years. The most active strains 1593 and strain 2362 which belong to serotype 5a5b (Charles *et al.,*1996, Delecluse *et al.,*2000). *Bacillus sphaericus* VCRC-B547 isolated from excreta of arid birds has shown higher toxicity against *Cx.quinquefasciatus, An.stephensi* and *Aed.aegypti* (Poopathi *et al.,* 2014). *Bs* tend to have higher residual activity than *Bti* in polluted waters. As a result, commercial product Vectolex (Abbott Laboratories) based on strain 2362 is marketed in many countries especially in polluted aquatic environments.

Limitations:

Though, *Bacillus sphaericus* remain effective against *Culex* spp., repeated application in the field for long term effect will lead to development of resistance in target species. Persistance and recycling potential of *B. sphaericus* are more achievable in polluted than in clear waters (Mulla *et al.,* 1984). Because, recycling is important phenomenon where toxin production continues during a period where several generations of target species are produced.

The lower sensitivity of *Bs* may result from the fact that the protein of the bacterium is enclosed in the exosporium, whereas the delta endotoxin of *Bti* is uncoated. It is possible that coated spore-crystal complex is more tolerant to UV light than the uncoated protein. This feature is also responsible for the slow mode of action of products based on *Bs* and its potential to persist under certain field conditions (Lacey, 2007). The biolarvicide formulation from *Bs* strain is reported to be less effective against *Anopheles culicifacies* and hardly effective against *Aedes aegypti (*Mittal, 2003). *Bs* is at high risk of selecting resistance

Table 2: Commercially available *Bacillus sphaericus* strains as mosquito larvicides.

Strain number	source of isolation	References
2297	Sri Lanka	Wickremesinghe RSB and Mendis CL, 1980
1593	Nigeria	Weiser J, 1984
2362	China	Liu EY *et al.,* 1989
VCRC-B547	Pondicherry	Poopathi *et al.,* 2014

Table 3 : List of *Pseudomonas* species reported as mosquitocidal bacterial strains

Pseudomonas species	Source of isolation	References
Pseudomonas fluorescens	Dead mosquito larva	Murty *et al.,*1994; Prabakaran *et al.,2003;* Sadanandane *et al.,* 2003; Prabakaran *et al.,* 2009; Prabakaran *et al.,* 2015; Pushpanathan and Selvaraj Pandian, 2008; Varun Rajan and Selvaj Pandian *et al., 2008,*Usharani and Paily, 2014; Lalithambika *et al.,* 2014; Athisayamary *et al.,* 2015; Mahamuni *et al.,* 2015;
Pseudomonas pseudomallei	Soil samples (Malaysia)	Lee and Seleena,1990.
Pseudomonas aeroginosa	*Guppies(Poectilia reticulate)*	Lysenko and Kuchera, 1968; Chadde,1992, De Barjac, 1989.
Pseudomonas frederiksbergiensis	contaminated soil (Saudi Arabia)	Ahmed *et al.,* 2014; 2015

in mosquito population. In fact, resistance to *B.s* has already been reported in field populations of *Culex* spp in China, Brazil, France and India (Sinegre *et al.,* 1994, Rao *et al.,* 1995, Silva Filha *et al.,* 1995,Yuan *et al* , 2000) with resistance levels in some areas of China reported as >20,000 fold. The potential key strategy for delaying resistance to mosquitocidal proteins is to use mixture of toxins that act at different targets within the insects (Writh *et al.,* 2005)

Resistance against *Bti* and *Bs*:

Due to continuous selection pressure and cross resistance, mosquito populations develop resistance against *Bs* binary toxin (Bin) both in the laboratory and field trials (Sinegre *et al.,* 1994). In Brazil, it was reported that the tenfold increase in resistant population found in open drains and covered cessepits in a small area where all the breeding sites were treated during two year period with a total of 37 treatments (Wirth *et al.,* 2000). But in case of *Bti* strains, they have been used for mosquitoes and Black flies for about 20 years, yet no resistance to this bacterium has been reported. In contrast to their sub species only low levels of resistance was observed in the laboratory experiments. The reason behind is selection of *Culex quinquefasciatus* with mutants of *B.thuringiensis sub species israelensis* that contained different combinations of its Cry proteins and Cyt1Aa delayed the evolution and expression of resistance to mosquitocidal Cry proteins (Wirth *et al.,* 2005)

Recombinant bacterial strains for vector control:

Commercial products such as VectoBac and Teknar based on *Bacillus thurigiensis subsp. israelensis(Bti),* VectoLex based on *Bacillus sphaericus* are most widely used as vector control products. Even though these products gain commercial success in developed countries but their high cost of fermentation, limited their use in developing countries. Lack of persistence due to settling of the spore-crystal complexes and narrow host range compared with chemical insecticides limited the usage of wild strains of *Bti* and *Bs* (Ohana *et al.,* 1987). Recombinant DNA technology pave the way for enhancing the synthesis of mosquitocidal proteins and by enabling new endotoxin combinations from different bacteria to be produced within single strain (Federici *et al.,* 2003). Recombinant *Bti* able to produce Cyt1A, Cry proteins and Bs binary toxin, in which Cyt1A delays resistance to insecticides (Wirth *et al.,* 2005). Higher specificity, environmental safety of the recombinants compared to synthetic insecticides with increased efficacy will provide these novel strains to be used in the future pest and vector control programmes (Park and Federici, 2009).

Clostridium bifermentans serovar Malaysia:

The first anaerobic mosquitocidal isolate, CH18 was isolated and identified from Mangrove swamp soil from Malaysia. Hence it was named as *C.bifermentans serovar Malaysia(Cbm)*.Another strain was isolated from the forest and reported as *C.bifermentans serovar pariba(Cbp)*(Seleena *et al.,* 1997).Both these strains were active against *Anopheles* larvae and in increasing level of susceptibility to *Aedes* and *Culex* species. Their toxicity remains similar to *Bti* strains but the toxic factors are different from *Bt.* Though the Clostridium species includes human pathogens, the safety of *Cbm* strains as potential bioinsecticide is highly considerable (Thiery *et al.,* 1992).

Pseudomonas species:

Pseudomonas species show remarkable and physiological versality, enabling colonization in diverse terrestrial and aquatic habitats (Palleroni, 1992). They are generally aerobic, gram-negative bacteria, ubiquitous in agricultural soils and are well adapted to grow in the rhizosphere. Stainer *et al., (*1966) conducted a fundamental study on the pseudomonas that result in an extensive phenotypic characterization in which the genus was subdivided into species and species into groups. *Pseudomonads* possess many traits that make them well suited as biocontrol and growth-promoting agents. Many biocontrol agents from *P*. *fluorescens* are well characterized for their ability to produce antimicrobial compounds, including 2,4-diacetylphloroglucinol (DAPG), phenazines, hydrogen cyanide and surfactants (Haas and De´fago, 2005).

Some exotoxins such as *Pseudomonas aeruginosa Migula* have been noted to be absorbed through the cuticle of insects and act on the haemolymph proteins. Exotoxins of microbial origin, including *Pseudomonas species* are also known to be toxic to larvae of mosquitoes as well as lepidopteran insects (Murty *et al.,* 1994).

The larvicidal effects of the culture supernatants of *Pseudomonas fluorescens*(MSS-1), originally isolated from deceased mosquito larvae reported to be active against *Culex quinquefasciatus, Anopheles stephensi,*

Aedes aegypti. A microbial formulation of *Pseudomonas fluorescens* (VCRC 426) was developed and formulated and tested against 4th instar larvae and pupae of three major vectors. *A. stephensi* was found to be most susceptible followed by *Culex quinquefasciatus* and *Aedes aegypti.* This was the first report that exotoxin remain effective against the pupae of the three species of mosquitoes at a very low concentration that of larvae (Prabhakaran *et al.,*2002). Field valuation of VCRC B426 formulation of *P.fluorescens* against *Culex quinquefasciatus* larvae and pupae showed 100% elimination of larvae and pupae at day1 after treatment and 80% reduction in pupal density (Sadanandane *et al.,*2003). The exotoxins produced by *Pseudomonas fluorescens* exhibited marked larvicidal and pupicidal activity against *A. aegypti and A. albopictus* (Pushpanathan and Selvaraj Pandian, 2008).

Binding of *Pseudomonas fluorescens* proteins to specific receptors plays an important role in the mode of action. It has been reported that binding of mosquitocidal proteins to the midgut region of treated larvae and pupae leads to considerable increase in the marker enzyme activity and Cytochrome C oxidase activity in th treated *Aedes albopictus* cell lines (Usharani and Paily, 2014). *Pseudomonas fluorescens Migula* (VCRCB426) produces secondary metabolite which is analysed and found as rhamnolipid. It is reported as first mosquito pupicidal compound which is found active against *culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti* (Prabakaran *et al.,* 2015). The major limitation of pseudomonads as biocontrol agents is their inability to produce resting spores which remain problematic in formulation of the product. Most of commercial products of *Bti* and *Bs* have their spore-crystal complex which have longer storage facility.

CONCLUSION

Collectively, arthopods are responsible for the transmission of vector-borne diseases both in human and animals. Over the past 30 years, there has been a global re-emergence of infectious diseases particularly vector-borne diseases with an increased frequency of epidemic transmission and expanding their geographical distribution. Many factors directly or indirectly contribute to emergence of vector borne diseases recently. Distribution of these diseases is determined by a complex dynamic of environmental and social factors such as globalization of travel and trade, unplanned urbanization, climate change etc which are having a significant impact on these diseases transmission in recent years These include climate change pattern, global trade, rapid unplanned urbanization, socioeconomic status, vector control programs which are highly influencing the current vector diseases epidemiology (Gubler, 2009). It has also been reported that the vectors in several countries has developed resistance to most of the highly effective class of insecticides. Hence, there remains a great challenge to control vector borne diseases. It is essential to develop a novel bioinsecticide which posses new mode of action, rapidly kills target species, high specificity and with commercial value. It is also essential to review about biocontrol agents for vector control and under laying fundamental capacities including technical expertise, stronger surveillance systems and better laboratory infrastructure facilities (WHO, 2014).

Conflicts of interest: The authors stated that no conflicts of interest.

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